Remarks

Claims 57, 58, 60-62, 64, and 76-86 were pending in the subject application. Accordingly, claims 57, 58, 60-62, 64, and 76-86 are currently before the Examiner for consideration. Favorable consideration of the pending claims is respectfully requested.

A Notice of Appeal to the Board of Patent Appeals and Interferences is submitted herewith.

Throughout the Remarks herein, where reference is made to the Declaration or the Sinden Declaration, the applicants refer to the Declaration under 37 C.F.R. §1.132 by Dr. Sinden, which accompanied the Amendment submitted on September 30, 2002. Likewise, reference to an Exhibit (*e.g.*, Exhibit D) refers to one of the Exhibits that accompanied the Declaration.

Claims 57, 58, 60-62, 64, and 76-86 have been rejected under 35 U.S.C. §112, first paragraph, as lacking sufficient written description. The applicants respectfully submit that the subject specification provides a sufficient written description of the claimed subject matter.

The applicants respectfully submit that the currently pending claims do not constitute new matter. Support for the phrase "a disorder associated with damage to, or loss of, brain cells in a mammal", which is recited in claims 57, 81, and 85, can be found throughout the subject application, and particularly at page 1, lines19-25, and page 5, lines 15-22, of the specification as originally filed. The specification teaches that when transplanted, "pluripotent neuroepithelial cells appear to respond to signals from the damaged or diseased brain by taking up a phenotype that is able to replace or compensate for functional deficits to which the damage or disease otherwise leads" and that "the phenotype of the differentiated cells may be the same as the phenotype of the damaged or lost cells, however, the differentiated cells may be of a different phenotype" and "the cells take up a phenotype that is capable of functionally integrating and compensating for the damaged or lost cells." At page 2, lines 14-17, the specification as originally filed states that the treatment may be carried out on any mammal.

Support for the phrase "wherein said cells are immortal prior to said transplanting and differentiate after said transplanting", which is recited in claim 57, can be found, for example, at page 5, lines 10-15 and lines 32-36, and page 6, lines 1-10 and 16-25, of the specification as originally filed. At page 5, lines 10-15, the specification teaches that "when conditionally immortal

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pluripotent neuroepithelial cells are implanted into a damaged brain the cells differentiate into the correct form of cell required to repair the damage and the differentiated cells are able to form the appropriate connections required to improve function" and that "conditionally immortal cells are cells which are immortal under certain permissive conditions but are not immortal under nonpermissive conditions". Thus, as explained at page 6, lines 6-10, of the specification, "if the conditions under which the cells are maintained are switched to nonpermissive conditions, the development of the cells is allowed to continue. If the correct conditions are provided the cells will continue to develop and will differentiate". As stated at page 6, lines 16-25, of the specification, "conditionally immortal cells have the advantages of immortal cells in that they are "frozen" in the desired stage of development, are easily maintained and multiply well when under permissive conditions but they may be used in transplants as long as the environment into which they are transplanted has nonpermissive conditions."

Support for the phrase "wherein said transplanting improves brain functions of said mammal", which is recited in claims 57 and 81, can be found, for example, at page 8, lines 1-5, which states that the transplanted cells are able to "differentiate in response to the local microenvironment, into the necessary phenotype or phenotypes to improve or restore function." Furthermore, at page 8, lines 21-27, the specification states that, preferably, the treatment will substantially correct a behavioral and/or psychological deficit. However, treatment according to the present invention may lead to improvement in function without complete correction.

Support for the phrase "a disorder associated with damage to, or loss of, brain cells in the hippocampus of said mammal", which is recited in claim 58, can be found, for example, at page 5, lines 15-22, page 13, lines 2-4, and Examples 5-9 at pages 22-29, of the specification as originally filed. At page 5, lines 15-22, the specification indicates that the implanted cells take up a phenotype that is capable of functionally integrating and compensating for damaged or lost cells. As described in Example 5 at page 22 of the specification, ischemic lesions were created in the CA1 area of the hippocampus of rats and restoration of spatial learning and memory was evaluated using the Morris water maze test. In Example 9, at pages 27-29, the specification describes assessment of post-mortem ischaemic brain damage, and indicates ischemia was present in the hippocampus of the animal model (see, for example, page 28, lines 1-2). As indicated at page 9, lines 29-35, and page

10, lines 1-9, the lesion-and-behavior model used in Examples 5-9 uses a technique of four-vessel occlusion (4 VO), "causing relatively circumscribed and specific damage to the CA1 pyramidal cells of the dorsal hippocampus, along with a cognitive deficit...".

Support for the phrase "wherein said transplanting improves cognitive function of said mammal", as recited in claims 57 and 81, can be found, for example, at page 8, lines 15-27, of the specification as originally filed, which states:

> After treatment the progress of the patient may be monitored using behavioral and/or psychological tests and/or, if desired, tests which monitor brain activity in selected areas of the brain. For example, tests for cognitive function may be performed before and after transplantation.
>
> Preferably, treatment will substantially correct a behavioral and/or psychological deficit. However, that may not always be possible. Treatment according to the present invention and with the cells, medicaments and pharmaceutical preparations of the invention, may lead to improvement in function without complete correction. Such improvement will be worthwhile and of value.

Furthermore, at page 9, lines 29-35, and page 10, lines 1-9, the specification teaches that the lesion-and-behavior model used in Examples 5-9 of the application causes a cognitive deficit due to loss of blood supply to the brain. The Morris water maze test was used to evaluate improvement in cognitive function.

Support for the treatment of humans, as recited in claims 82 and 86, can be found throughout the specification, where it is clear that treatment of humans was contemplated. For example, at page 2, lines 14-17, the specification as originally filed indicates that "treatment may be carried out on any mammal but the present invention is especially concerned with the treatment of humans, especially treatment with human cells, and with human cells and cell lines". At page 4, lines 1-3, the specification as originally filed indicates "a further aspect of the invention provides for conditionally immortal, pluripotent, neuroepithelial cells for therapeutic use, especially in humans."

Contrary to the statements at page 6 of the Office Action, the applicants respectfully submit that the specification as a whole does support using neuroepithelial cells other than hippocampal neuroepithelial cells. As indicated at page 13, lines 5-7, of the specification as filed, "the part of the fetal brain from which the neuroepithelial cells are taken and the precise time (stage and development) may vary". Furthermore, as indicated at page 12, lines 33-36, and page 13, lines 1-4, uses of cells according to the invention are not limited to repair of the particular type of damage

modeled in Examples 6, 7, and 8; rather, "transplantation into any area of the brain is envisaged with consequent improvement in function". Examples of diseases or conditions which result in behavioral and/or psychological deficits which may be treated in accordance with the present invention are set forth at page 3, lines 1-14, of the specification as originally filed.

As the Examiner is undoubtedly aware, the subject matter of a claim need not be described literally (*i.e.*, using the same terms or *in haec verba*) in order for the disclosure to satisfy the written description requirement. "It is not necessary that the application describe the claim limitations exactly,...but only so clearly that persons of ordinary skill in the art will recognize from the disclosure that appellants invented processes including those limitations". *In re Wertheim*, 191 USPQ 90, 96 (C.C.P.A. 1976). "*Ipsis verbis* disclosure is not necessary to satisfy the written description requirement of section 112. Instead, the disclosure need only reasonably convey to persons skilled in the art that the inventor had possession of the subject matter in questions." *Fujikawa v. Wattanasin*, 39 USPQ2d 1895, 1904 (Fed. Cir. 1996), quoting from *In re Edwards*, 196 USPQ 465, 467 (CCPA 1978). The applicants respectfully submit that the specification provides adequate support for the claimed subject matter, describing the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventors had possession of the claimed subject matter, which is all that is required to satisfy the written description requirement of 35 U.S.C. §112, first paragraph. The claimed subject matter does not represent new matter.

The Office Action states that the subject specification teaches using mouse cells to restore cognitive function and suggests the use of human cells isolated at about eight weeks gestation. The Office Action cites the Gray *et al.* publication as showing that fully differentiated hippocampal cells not yet having axons are essential to the invention, that the isolation of suitable cells must be taken at fifteen weeks gestation, and that this is essential to obtain the required amount of differentiation. However, the passage in the Gray *et al.* publication relied on by the Examiner concerns an overview of conventional transplantation methods using fully differentiated cells, not the pluripotent nestin-positive cells used in the method of the invention. When transplanting differentiated cells, it is clearly important that the correct phenotype of cell be selected for transplantation. Therefore, the applicants respectfully submit that the Examiner's interpretation of the teaching of Gray *et al.* is incorrect.

In contrast to differentiated cells, the cells of the present invention are pluripotent and have the ability to differentiate into different phenotypes, depending on external factors. Upon transplantation, the pluripotent cells are stimulated into differentiating into the desired phenotype (see page 5, lines 10-24, of the specification as originally filed). Therefore, controlling differentiation is not an issue for the conditionally immortal pluripotent neuroepithelial cells used in the methods of the subject invention.

At page 10, the Office Action states that the applicants do not provide an adequate written description for the human equivalent of the mouse, nestin-positive cells capable treating cognitive function. The subject specification teaches that the pluripotent cells used in the claimed method should be isolated early enough in the developmental pathway that they retain the ability to differentiate into the desired brain cell phenotypes (page 13, lines 7-11). It is well known by those skilled in the art that the plasticity (*e.g.*, pluripotency) of embryonic cells is generally inversely related to the age of embryonic development. Therefore, if nestin-positive pluripotent cells are obtainable from a human at 12 weeks gestation, it is at least as likely, if not more likely, that the cells would be obtainable from a human at an earlier stage of development, *e.g.*, 8 weeks gestation. As indicated at page 13, lines 5-7 of the specification, the region of the brain from which neuroepithelial cells are obtained and the precise time (stage and development) they are obtained may vary. The Declaration under 37 C.F.R. §1.132 by Dr. Sinden, which accompanied the Amendment submitted on September 30, 2002, shows that pluripotent nestin-positive neuroepithelial cells can be obtained from human fetal cortex at 12 weeks gestation (see paragraph 9 and Exhibit D of the Declaration).

Thus, page 13, lines 5-16, of the specification highlights 8 weeks as an example of when such pluripotent cells can be isolated. The human cells can also be isolated at 12 weeks (as demonstrated by Exhibit D) and even later, depending on the brain region. As will be appreciated by one of ordinary skill in the art, the hippocampus and cortex are properly identifiable as anatomical structures at approximately 10-12 weeks, which is why the human cells were isolated at the 12-week gestation period. The cells described in Exhibit D were taken from the cortex because the cells are readily obtainable in large numbers from this brain region. The success of the experiment described in Exhibit D using cortex cells supports the teaching of the specification, *i.e.*, that pluripotent neuroepithelial cells other than hippocampal cells are capable of improving a brain disorder, such as

cognitive deficit. Once obtained, cells can then be screened *in vitro* to verify their ability to differentiate upon transplantation, as taught at page 13, lines 17-18, and Example 4, at pages 20-21 of the specification. The Examiner has provided no reasons to doubt that the pluripotent cells used in the claimed methods can be obtained from more than one region of the brain or at more than one gestational stage.

At page 10 of the Office Action, the Examiner observes that the human cells used in the experiment described in Exhibit D express both nestin (intermediate filament marker) and musashi l, and concludes that this information would have been required for one of ordinary skill in the art to obtain the cells for transplantation. As indicated in Exhibit D, nestin and musashi 1 are both phenotypic markers for neuroepithelial stem cells. The musashi 1 marker was more recently identified than nestin and, hence, merely confirms the neural epithelial status of the cells, as determined by nestin expression. Submitted herewith for the Examiner's consideration are Kawaguchi *et al.* (*Molecular and Cellular Neuroscience*, 2001, 17:259-273) and Sakakibara *et al.* (PNAS, 2002, 99(23)15194-15199). The Sakakibara *et al.* publication indicates that musashi is a binding protein that is expressed in neural precursor cells, which can be used to identify precursor cells. As indicated in the abstract of the Sakakibara *et al.* publication, the musashi family of proteins are evolutionarily conserved across species. In mammals, musashi 1 and musashi 2 are strongly coexpressed in neural precursor cells, including CNS stem cells. Likewise, the Kawaguchi *et al.* publication demonstrates that nestin is expressed in neural precursor cells. Use of musashi is equivalent to use of nestin. Thus, one of ordinary skill in the art would appreciate that musashi 1 expression does not represent a characterizing feature of the cells that had to be identified before the invention could be carried out; demonstrating the expression of nestin by the cells, as taught in the specification, is sufficient. The mere fact that the human cells were further characterized and it was determined that the cells expressed musashi 1, in addition to nestin, should not disqualify the experimental results and accompanying Declaration by Dr. Sinden as probative evidence in the determination of written description and enablement under 35 U.S.C. 112, first paragraph.

The Office Action also cites the Renfranz publication for teaching that differentiation of nestin-positive pluripotent cells is varied. The Renfranz publication teaches the establishment of a differentiated cell line derived from embryonic precursor cells. Because the cells are pluripotent,

they have the ability to differentiate into different types of neural cells, depending on environmental factors. This is consistent with the properties of the pluripotent neuroepithelial cells used in the method of the invention. As taught at page 5, lines 10-24, of the subject specification, upon transplantation, the pluripotent cells are stimulated into differentiating into different phenotypes that are capable of functionally integrating and compensating for the damaged or lost cells.

The Office Action also cites the Villa *et al.* publication as showing that properties identifying human neural stem cells are not well understood. The applicants respectfully submit that the Villa *et al.* publication supports the written description of the claimed subject matter in that it shows that one of ordinary skill in the art can obtain cells having the properties that the specification teaches are desirable. The Villa *et al.* publication is concerned with defining the optimal conditions for preparing suitable pluripotent cells and makes various statements that genetically-modified cells provide the most convenient method. The cells used in the Villa *et al.* publication are taken at approximately ten weeks gestation. Furthermore, the Villa *et al.* publication makes it clear that suitable cells can be defined in terms of nestin expression and pluripotency. These are properties of the cells that are taught in the specification and recited in the claims of the subject application. Although there is a statement in the Villa *et al.* publication that properties identifying a human neural stem cell are not well understood, this does not mean that a human neuroepithelial pluripotent cell cannot be identified. Clearly, Villa *et al.* and others in the art have done so. Although the Villa *et al.* publication was not publicly available at the time of filing, it demonstrates that the subject specification contained all the information necessary for one of ordinary skill in the art to carry out the invention.

The applicants respectfully submit that the subject specification reasonably conveys to those of ordinary skill in the art that the inventors were in possession of the claimed subject matter. In view of the foregoing remarks, reconsideration and withdrawal of the rejection under 35 U.S.C. §112, first paragraph, is respectfully requested.

Claims 57, 58, 60-62, and 76-86 have been rejected under 35 U.S.C. §112, first paragraph, as non-enabled. The applicants respectfully submit that the claimed invention is fully enabled by the subject specification.

The Scheffler *et al.* publication (*Trends in Neurosci.*, Vol. 22, pg. 348-357, 1999) has been cited in the outstanding Office Action and earlier Actions as showing that it was "unpredictable" how to target particular areas of the brain when transplanting neural cells. However, the relevant passage in the Scheffler *et al.* publication refers to a study that used transplanted post-natal and adult neurons, and did not relate to the transplantation of conditionally immortal pluripotent neuroepithelial cells. As indicated in the Sinden Declaration, "one of the great advantages of the present invention is that it is not necessary to target particular areas of the brain to correct cell damage." This is emphasized at various points throughout the subject specification, as well (see, for example, page 5, lines 10-31). As Dr. Sinden explains in his Declaration,

> previously, it was thought that to treat damage in a developed postnatal or adult brain, it was necessary to use tissue/cells derived from the same area as that damaged. Importantly, prior to our invention, even if the cells to be transplanted were taken from a fetus, ... the cells would typically be committed to a particular phenotype. Moreover, prior to our work, there was no selection of nestin-positive, pluripotent cells, or genetic modification of the cells to confer conditional immortality such that the cells would be immortal prior to transplantation but differentiate subsequent to transplantation.

The inventors realized that, "surprisingly, transplanting cells that were selected to retain a nestin-positive, pluripotent, conditionally immortal phenotype resulted in the repair of damage, and this was independent of the site of damage", as indicated in Dr. Sinden's Declaration. Accordingly, using the cells of the claimed invention permits treatment at different sites of damage with a single cell line, which is selected on the basis of its nestin-positive, pluripotent characteristics, and is genetically modified to be conditionally immortal.

In contrast to the observations made in the Scheffler *et al.* publication, as stated above, the applicants have shown that the targeting of cells is not necessary using the cells of the subject invention. The subject specification teaches that the cells migrate to areas of damage after transplantation, and become integrated in the damaged areas, effecting repair. As explained by Dr. Sinden in his Declaration, "this ability of the cells to migrate (which we were the first to observe) is an inherent feature of the cells; therefore, the difficulties identified in the Scheffler *et al.* publication will not be experienced when using nestin-positive, pluripotent neuroepithelial cells that have been genetically modified to be conditionally immortal."

It is well settled patent law that an applicant's statements must be accepted as true unless the Patent Office can provide evidence to doubt the truth of those statements. *In re Marzocchi*, 169 USPQ 367 (CCPA 1971). The Examiner has not provided acceptable reasoning for doubting the statements within the specification or the Declaration by Dr. Sinden. The record is replete with evidence supporting the truth of the specification's teachings that the cells of the invention migrate to areas of damage after transplantation, become integrated within the damaged brain, and achieve repair.

At page 17 of the Office Action, the Examiner states "a mere statement that the cells do not require targeting particular areas of the brain without evidence or scientific reasoning is inadequate to overcome the rejection". The applicants respectfully submit that more than "a mere statement" has been made of record in support of the ability of the recited cells to migrate to different areas of the brain, integrate, and effect repair. Submitted with the Sinden Declaration as Exhibit B was a copy of U.S. Patent Application Publication No. 2002/0037277 (now U.S. Patent No. 6,569,421). The example at pages 2-5 of the published application clearly demonstrates migration of the cells, and also shows that cells from one region of the brain (hippocampal region) can repair damage to a different area of the brain, such as cortex and basal ganglia.

The cells utilized in the example of Exhibit B are nestin-positive, pluripotent neuroepithelial cells that have been genetically modified to be conditionally immortal, as recited in the currently pending claims. The outstanding Office Action indicates that "no evidence can be found that the cells in Exhibit B were prepared the same as those described in the specification". The applicants respectfully draw the Examiner's attention to paragraph 0030 at column 2 of the '277 publication, which states "conditionally immortal pluripotent neuroepithelial cells from the MHP36 clonal cell line were prepared as disclosed in WO-A-97/10329, which is the published PCT application (PCT/GB96/02251) to which the subject application claims priority. The MHP36 clonal cell line is used in the Examples in the subject application.

As indicated in the Sinden Declaration, regarding Exhibit B (the '277 publication), "compelling evidence of extensive migration is presented at page 4, paragraph 0047, which indicates that contralaterally grafted cells 'migrated across the midline to the opposite side of the brain (emphasis added)'." The outstanding Office Action indicates that page 4, paragraph 0047, of Exhibit

B "merely discusses migration and does not state adequate numbers of cells migrated to the site of tissue damage such that the cognitive deficit was treated." Exhibit B indicates that migration of implanted cells was extensive (see paragraphs 0047 – 0049). The issue of the number of migrating cells in the experiment is of limited relevance, since functional recovery was confirmed. The applicants respectfully draw the Examiner's attention to pages 4 and 5, paragraphs 0050 – 0053, of Exhibit B (the '277 publication). The stated purpose of the experiments was to determine whether grafts of MHP36 cells, from a conditionally immortalized clonal line, would promote functional recovery from stroke damage when placed in the intact hemisphere contra-lateral to the infarct cavity (see paragraph 0050 at page 4 of Exhibit B). As indicated in paragraph 0050 and 0051 of Exhibit B, "the findings indicate that both sensorimotor and motor asymmetries were normalized in rats with grafts initially sited in the intact hemisphere...The evidence for recovery of sensorimotor and motor functions is robust, because improvements were seen over an extended time period."

The applicants note that the currently pending claims recite that transplanting the cells improves brain function (*e.g.*, cognitive function) in the mammal. Thus, it would be understood by those skilled in the art that the number of cells transplanted would be an amount effective to achieve the recited improvement in function. Guidance regarding the number of cells to be transplanted is provided page 8, lines 28-34, of the specification as originally filed.

The Snyder *et al.* patent (U.S. Patent No. 6,528,306), which was submitted previously, describes the migratory properties of neural stem cells and demonstrates that the cells can be maintained in culture in an undifferentiated state, with differentiation occurring upon transplantation. The Snyder *et al.* patent was not publicly available at the priority date of the subject application; however, it demonstrates that the subject specification contained all the information necessary for the skilled person to carry out the invention. The background section of the Snyder *et al.* patent cites several early scientific papers reporting the behavior exhibited by neural stem cells upon transplantation. For example, at column 1, lines 44-56, the Snyder *et al.* patent indicates that neural stem cells are extremely plastic and can migrate and differentiate "in a temporally and regionally appropriate manner ..., responding similarly to local microenvironmental cues for their phenotypic determination and appropriately differentiating into diverse neuronal and glial cell types." Furthermore, at column 1, lines 40-43, the Synder *et al.* patent cites several early publications as

demonstrating the behavior of pluripotent neural stem cells following transplantation, including their ability to interact with host cells and differentiate appropriately.

The applicants submit that, given the benefit of the specification's disclosure, a person of ordinary skill in the art could readily identify and use nestin-positive, pluripotent neuroepithelial cells as recited in the claims. Evidence of the ability to use a variety of pluripotent, nestin-positive neuroepithelial cells to treat various tissues is provided within Exhibit D, as described above. Exhibit D describes an experiment carried out using human nestin-positive pluripotent neuroepithelial cells derived from the human fetal cortex, to treat damage associated with the basal forebrain. These cells were genetically modified to be conditionally immortal, as recited in the currently pending claims. As indicated above, the applicants respectfully submit that the teachings of the specification and Exhibit D are consistent, and the human nestin-positive neuroepithelial cells described in Exhibit D correlate with the teachings of the subject specification as originally filed. As indicated at page 13, lines 5-7, the region of the brain from which neuroepithelial cells are obtained and the precise time (stage and development) they are obtained may vary.

The Sinden *et al.* publication (*Neuroscience*, 81:599-608, 1997) has been cited in the outstanding Office Action and previous Actions as suggesting that CA1 cells derived from the hippocampus must be used to repair damaged CA1 hippocampal tissue. The applicants submit that the cited portion of the Sinden *et al.* (1997) reference is merely characterizing the prior art. As indicated in the Sinden Declaration, "the statement referred to by the Reviewer within the Sinden *et al.* publication (of which I am the first author), is made with respect to a previous study that used primary cells that were mature, differentiated or committed CA1 cells, and not the conditionally immortal, pluripotent, nestin-positive, neuroepithelial cells that are used in the method of our invention" (emphasis added).

As indicated in the Sinden Declaration, "provided the neuroepithelial cells are nestin-positive and retain the ability to differentiate into the specified phenotypes in response to environmental signals, they are appropriate for use in the present invention." The Scheffler *et al.* publication does not provide any reason to doubt that one of ordinary skill in the art, having the benefit of the specification's disclosure, can determine what is, and what is not, an appropriate pluripotent neuroepithelial cell for use in the subject invention.

While the applicants acknowledge that some experimentation and screening may be required to isolate human, pluripotent, nestin-positive neuroepithelial cells, the court in *In re Wands* has stated

Enablement is not precluded by the necessity for some experimentation such as routine screening. However, experimentation needed to practice the invention must not be undue experimentation. The key word is 'undue' not 'experimentation'.

> The determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988).

The cells recited in the claimed methods are positive for the progenitor cell marker, nestin. As indicated in the Sinden Declaration, "nestin-positive cells can be readily identified using immunocytochemistry," as described at pages 20 and 21 of the subject patent application, or by other techniques known to those of ordinary skill in the art. Furthermore, Example 4 of the specification describes an *in vitro* screening method for determining the pluripotency of the cells *in vivo* (see page 13, lines 23-26). The experimentation and screening required to obtain the necessary pluripotent, nestin-positive neuroepithelial cells are standard and routine in the art. Thus, the applicants respectfully submit that the subject specification provides adequate guidance for the skilled person to identify and use appropriate cells, without resort to undue experimentation.

The Action also indicates that the subject specification does not provide sufficient guidance for conferring conditional immortality to cells (*e.g.*, via the temperature sensitive oncogene (tsA58)). As taught in the specification, such conditionally immortal cells can be readily prepared by transduction of an oncogene into a cell (see, for example, page 6 of the specification). As taught at page 7, lines 1-7, of the specification, the use of non-human transgenic animals is but one method for obtaining conditionally immortalized cells. Conditional immortality is described on page 5, last paragraph, and pages 6 and 7 of the specification, and it is clear that the cells remain immortal (undifferentiated and continuously dividing) under one set of conditions, but can be induced to mature and differentiate (losing immortality) by a change in conditions. The Frederiksen *et al.*

publication (*Neuron*, Vol. 1, 439-448, 1988) and Jat *et al.* publication (*Proc. Natl. Acad. Sci. USA*, 88:5096-5100, June 1991), which accompanied the Amendments submitted on September 30, 2002 and March 20, 2003, respectively, show that methods for achieving conditional immortality using, for example, the temperature-sensitive SV40 oncogene, were known in the art even in 1988. The background section of the Snyder *et al.* patent also highlights methods (both epigenetic and genetic) for immortalizing cells that are not dependent on the large T antigen temperature-sensitive oncogene (see column 1, lines 31-43 of the Snyder *et al.* patent). Therefore, the applicants respectfully assert that the specification fully enables human, pluripotent, nestin-positive neuroepithelial cells that have been genetically modified to be conditionally immortal, as recited in the currently pending claims.

The human cells used in the experiment described in Exhibit D express both nestin (intermediate filament marker) and musashi 1. As indicated in Exhibit D, nestin and musashi 1 are both phenotypic markers for neuroepithelial stem cells. The musashi 1 marker was more recently identified than nestin and, hence, merely confirms the neural epithelial status of the cells, as determined by nestin expression. As indicated above with regard to the rejection for lack of written description, submitted herewith for the Examiner's consideration are the Kawaguchi *et al.* and Sakakibara *et al.* publications. The Sakakibara *et al.* publication indicates that musashi is a binding protein that is expressed in neural precursor cells, which can be used to identify precursor cells. As indicated in the abstract of the Sakakibara *et al.* publication, the musashi family of proteins are evolutionarily conserved across species. In mammals, musashi 1 and musashi 2 are strongly coexpressed in neural precursor cells, including CNS stem cells. Likewise, the Kawaguchi *et al.* publication demonstrates that nestin is expressed in neural precursor cells. Use of musashi is equivalent to use of nestin. Thus, detection of musashi 1 expression, in addition to detection of nestin expression, is not "essential" or "required". One of ordinary skill in the art would appreciate that musashi 1 expression does not represent a characterizing feature of the cells that had to be identified before the invention could be carried out; demonstrating the expression of nestin by the cells, as taught in the specification, is sufficient. The mere fact that the human cells were further characterized and it was determined that the cells expressed musashi 1, in addition to nestin, should not disqualify the experimental results and accompanying Declaration by Dr. Sinden as probative evidence in the determination of written description and enablement under 35 U.S.C. 112, first

paragraph. The applicants respectfully submit that the experiment described in Exhibit D is commensurate in scope with the claimed invention, *i.e.*, bears a reasonable correlation to the scope of the claimed invention.

The applicants respectfully submit that a person skilled in the art, having the benefit of the specification's disclosure, could readily make and use the claimed invention without resort to undue experimentation. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. §112, first paragraph, is respectfully requested.

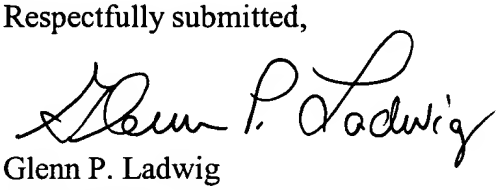
Claims 57, 58, 60-62, and 76-86 have been provisionally rejected under the judicially created doctrine of "obviousness-type" double patenting as being unpatentable over claims of copending U.S. Application Nos. 10/342,692 and 10/376,119. The applicants have submitted herewith Terminal Disclaimers executed by the undersigned attorney of record and in compliance with the requirements of 37 CFR §1.321(c), which obviates the rejections based on U.S. application Nos. 10/342,692 and 10/376,119. These Terminal Disclaimers are being submitted solely to expedite prosecution of the subject application to completion and should not be construed as an admission that the claimed subject matter is obvious over the claims in the cited patents, or vice-versa. Accordingly, reconsideration and withdrawal of the "obviousness-type" double patenting rejections is respectfully requested.

In view of the foregoing remarks and amendments to the claims, the applicants believe that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§ 1.16 or 1.17 as required by this paper to Deposit Account 19-0065.

The applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,



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Attachments: Petition and Fee for Extension of Time
Two (2) Terminal Disclaimers
Notice of Appeal
Sakakibara *et al.* publication (2002)
Kawaguchi *et al.* publication (2001)

RNA-binding protein Musashi family: Roles for CNS stem cells and a subpopulation of ependymal cells revealed by targeted disruption and antisense ablation

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Homologues of the Musashi family of RNA-binding proteins are evolutionarily conserved across species. In mammals, two members of this family, Musashi1 (Msi1) and Musashi2 (Msi2), are strongly coexpressed in neural precursor cells, including CNS stem cells. To address the *in vivo* roles of *msi* in neural development, we generated mice with a targeted disruption of the gene encoding Msi1. Homozygous newborn mice frequently developed obstructive hydrocephalus with aberrant proliferation of ependymal cells in a restricted area surrounding the Sylvius aqueduct. These observations indicate a vital role for *msi1* in the normal development of this subpopulation of ependymal cells, which has been speculated to be a source of postnatal CNS stem cells. On the other hand, histological examination and an *in vitro* neurosphere assay showed that neither the embryonic CNS development nor the self-renewal activity of CNS stem cells in embryonic forebrains appeared to be affected by the disruption of *msi1*, but the diversity of the cell types produced by the stem cells was moderately reduced by the *msi1* deficiency. Therefore, we performed antisense ablation experiments to target both *msi1* and *msi2* in embryonic neural precursor cells. Administration of the antisense peptide-nucleotides, which were designed to specifically down-regulate *msi2* expression, to *msi1*$^{-/-}$ CNS stem cell cultures drastically suppressed the formation of neurospheres in a dose-dependent manner. Antisense-treated *msi1*$^{-/-}$ CNS stem cells showed a reduced proliferative activity. These data suggest that *msi1* and *msi2* are cooperatively involved in the proliferation and maintenance of CNS stem cell populations.

During mammalian CNS development, neurons and glial cells are thought to be generated from common neural precursor cells (CNS stem cells) located in the periventricular area (1). The molecular basis for the maintenance of this cell population is, however, largely unknown. The recent discovery of neural RNA-binding proteins raises the possibility that the development of neural cells from their precursors may be controlled by posttranscriptional gene regulation, including mRNA stabilization, splicing, or translational control. Musashi1 (Msi1) and Musashi2 (Msi2) are RNA-binding proteins that are characterized by two RNP-type RNA recognition motifs (RRMs) and show remarkable similarity to one another, both in their primary structures and their RNA-binding specificities *in vitro* (2–4). These two molecules seem to define the Msi family of RNA-binding proteins, which is evolutionarily conserved across different species. In mammals, Msi1 and Msi2 expression is developmentally regulated. Our previous studies revealed that Msi1 and Msi2 are coexpressed predominantly in proliferating embryonic pluripotent neural precursors (2, 4–6), as well as in cell populations that are believed to be the source of postnatal and adult CNS stem cells (3, 6). In the cerebral cortex, the expression of Msi1 and Msi2 is rapidly down-regulated in newly generated postmitotic neurons (2), with the exception of some GABAergic interneurons that continue to express Msi2 exclusively (4). Although the molecular functions of the Msi family members remain obscure, their expression profiles suggest that they may play similar roles in the development and maintenance of CNS stem cells through posttranscriptional gene regulation (6).

In the present study, we used targeted disruption of the *msi1* gene in mice and antisense ablation of Msi2 to address the roles of these proteins during development. The results suggested that the Msi family genes have critical functions in restricted cell populations, including CNS stem cells.

Materials and Methods

Gene Targeting. Genomic DNA fragments of *msi1* gene (1.5-kb *Not*I–*Sal*I fragment for the left arm, and 9.4-kb *Hin*dIII–*Sal*I fragment for the right arm) were isolated from 129/Sv genomic DNA library and inserted into the targeting vector, which contained a 1.8-kb G418 resistance (*neo*) cassette and the diphtheria toxin A-fragment (DT-A) cassette. This targeting construct was designed to delete 4.2 kb of genomic DNA, including a 267-bp exonic sequence spanning the translational initiation codon and the first RRM, and to replace them with the *neo* cassette in the same transcriptional orientation as *msi1*. Correctly targeted ES cells, chimeric males, and the progeny of heterozygous intercrosses were genotyped by Southern blot or PCR analysis, using WT and mutant allele specific primers (primer sequences available on request). After seven to eight generations of backcrossing heterozygous mutants and C57BL/6 females, the F_8 or F_9 progeny of heterozygous intercrosses was used for histological and *in vitro* culture analyses. Histological analyses were performed as described (2, 4).

Antisense Peptide Nucleic Acids (asPNA). The asPNAs were custom synthesized, purified, and analyzed by PE Biosystems. The sequences of the *msi2* asPNA corresponded to the translation initiation codon (PNA1, 5′-CTCCATAGCGGAGCC-3′-Lys) or the coding region (PNA2, 5′-ACCTAATACTTTATCT-3′-Lys).

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Abbreviations: Msi1, Musashi1; Msi2, Musashi2; VZ, ventricular zone; SVZ, subventricular zone; SCO, subcommissural organ; SFC, sphere-forming cell; RRM, RNA recognition motifs; asPNA, antisense peptide nucleic acids; CC, corpus callosum; CSF, cerebrospinal fluid; BrdU, 5-bromodeoxyuridine.

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These two asPNAs had similar effects on neurosphere formation, and PNA1 was used throughout the experiments in this report. A scrambled PNA (5′-AACTCTTATATCTCTA-3′-Lys), which did not match significantly any other known RNA or DNA sequence, was used for the control.

Neurosphere Culture. The basic culture medium, containing 20 ng/ml epidermal growth factor (Sigma) and 10 ng/ml FGF2 (R & D Systems), and the procedures for the neurosphere formation and differentiation assays were as described (7). Briefly, cells from the anterior halves of the E14.5 telencephalon were used for primary sphere formation (1×10^5 cells per ml). The indicated amount (0–10 μM) of asPNA was added to the culture medium when the primary spheres were dissociated and replated for secondary sphere formation (500 cells per 200 μl per well, 96-well plate). The numbers of secondary spheres were counted 4 days later (4 div). Cells used for semiquantitative RT-PCR and immunocytochemistry for Msi2 or nestin (Rat401) were harvested after 24 h (1 div) of treatment with the asPNA. To assess cell proliferation within the asPNA-treated spheres, 2 μM BrdU (5-bromodeoxyuridine, Sigma) was administrated to the cultures at 2 div After 24 h of incubation, spheres were trypsinized, dissociated, and plated onto polyL-lysine-coated coverslips for 3 h, then processed for immunocytochemistry using anti-BrdU antibody (Sigma).

Semiquantitative RT-PCR. Cells incubated with or without asPNA (10 μM) for 24 h were collected (1.5×10^5 cells). Semiquantitative RT-PCR was carried out using the primer sets for *msi2* (5′-GTCTGCGAACACAGTAGTGGAA-3′ and 5′-GTAGCCTCTGCCATAGGTTGC-3′; 340 bp), *AUF1* (5′-ACTGCACTCTGAAGTTAGATCCTA-3′ and 5′-TGTAGCTATTTTGATGTCCACCTC-3′; 534 bp), *hnRNPA1* (5′-ATGGCTAGTGCTTCATCCAGTCA-3′ and 5′-CTGTGCTTGGCTGAGTTCACAAA-3′; 508 bp), *hnRNPC1/C2* (5′-AGTGGATTCAATTCGAAGAGTGGA-3′ and 5′-GATGACCTGGTGTTACTTTCATCT-3′; 513 bp), *NKT* (5′-TAAGCTGAGTATAGAGGTGCT-3′ and 5′-CATTCACTAGGATACAGATGC-3′; 332 bp), and *g3pdh* (5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′; 452 bp).

Results

msi1$^{-/-}$ Mice Develop Obstructive Hydrocephalus with Ependymal Abnormalities. Targeted disruption of the *msi1* locus in ES cells was performed by replacing four exons, including the translation initiation codon, with a *neo*-resistance gene cassette (Fig. 1 *A* and *B*). Interbreeding of the heterozygous mutant (*msi1*$^{+/-}$) mice yielded homozygous mutant (*msi1*$^{-/-}$) pups with the expected Mendelian ratio, indicating that Msi1 is not essential for embryonic viability. The absence of Msi1 expression in embryonic and neonatal brains in homozygous animals was confirmed by immunoblot analysis (Fig. 1*C*) using an anti-Msi1 monoclonal antibody.

Histological analyses showed that *msi1*$^{-/-}$ embryos (E10–E17) exhibited normal cortical development. Neuroepithelial cell proliferation in the ventricular zone (VZ) was not perturbed, as determined by light microscopy and BrdU incorporation analysis (not shown). Newborn *msi1*$^{-/-}$ pups were normal in size and appearance for the first postnatal week. However, by 1–2 postnatal weeks, 70–80% of the *msi1*$^{-/-}$ animals developed hydrocephalus with progressive dilation of the lateral ventricles and an enlarged, domed cranium (Fig. 2 *E–I*). In addition, a small population of *msi1*$^{-/-}$ mice (5–10%) exhibited Probst's bundle (8), an entangled axon tract in the corpus callosum (CC), because of errors in the projection of commissural fibers across the midline in the forebrain (Fig. 2 *A–D*). This callosal agenesis was observed at E17, when normal CC axons have arrived at the midsagittal plane (not shown). Agenesis or hypoplasia of the CC

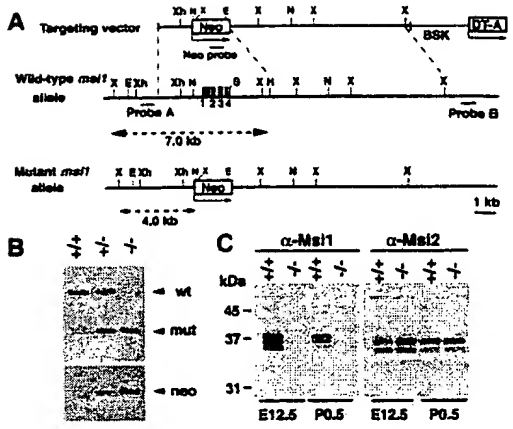

Fig. 1. Targeting strategy, germ-line transmission, and expression analysis of the *msi1* gene. (*A*) Organization of the targeting vector, the *msi1* gene, and the allele resulting from homologous recombination. Four exons (black boxes) of the *msi1* allele containing the initiation codon were replaced with a Neo cassette. A 0.3-kb *Sau*3AI fragment (probe A) was used to screen for recombinant alleles, and the sizes of the recombinant and WT fragments after *Xba*I digestion are shown (broken lines). X, *Xba*I; E, *Eco*RV; Xh, *Xho*I; Not, *Not*I; S, *Sal*I; H, *Hin*dIII; BSK, plasmid vector. (*B*) Germ-line transmission was confirmed by Southern blot analysis of *Xba*I- or *Eco*RV-digested tail DNA from a litter of F_6 mice using probe A or the Neo probe, respectively. (*C*) Immunoblot analysis of brain lysates from E12.5 and P0.5 mice using anti-Msi1 or anti-Msi2 antibodies. Genotypes are indicated.

with Probst bundles is frequently found in hereditary hydrocephalus in humans (X-linked hydrocephalus) (9) and in several targeted and spontaneous mouse mutants, e.g., *hyh* (10) and E2F-5 (11), suggesting that complex multigenic developmental pathways are disrupted in the *msi1*$^{-/-}$ mice.

Pathologic examination of the *msi1*$^{-/-}$ adult brains revealed dilation of the lateral ventricles and the third ventricle, thinning of the cerebral cortices, hypoplasia of the septa, disruption of the white and gray matters accompanied by intracerebral hemorrhage, edema, and necrosis of the periventricular parenchyma. The fourth ventricle was not dilated in *msi1*$^{-/-}$ mice. The olfactory bulb, cerebellum, thalamus, medulla, and hippocampus were not primarily affected, although they appeared to be compressed by the elevated intracranial pressure (Fig. 2 *F* and *I*). The dilation of the lateral ventricles was frequently accompanied by cavitation in the subventricular zone (SVZ) (Fig. 2*O′*). The areas surrounding the anterior horns of the lateral ventricles were occasionally denuded of ependymal cells or had a disrupted ependymal lining (Fig. 2*O′*). Analyses of P0 forebrain sections by BrdU labeling, TUNEL staining, and immunohistochemistry using cell-type-specific markers failed to demonstrate significant changes in the number of apoptotic cells, differentiated neurons, or proliferating precursor cells in the *msi1*$^{-/-}$ SVZ before the onset of ventricular dilation (not shown). The *msi1*$^{-/-}$ mice with severe hydrocephalus demonstrated ataxic gate and dehydration, and they eventually died within 1–2 months after birth. Moderate cases exhibited the dilated lateral ventricles and cavitation of the septum pellucidum (Fig. 2*H*) but survived into adulthood.

In the *msi1*$^{-/-}$ mice, abnormal proliferation and polyposis were observed in ependymal cells surrounding the Sylvius aqueduct, as well as in the subcommissural organ (SCO), an ependymal gland located at the entrance of the cerebral aqueduct that secretes glycoproteins into the cerebrospinal fluid (CSF) (12) (Fig. 2 *K* and *L*). These glycoproteins are known to aggregate in the CSF to form a fibrous structure (Reissner's fiber) along the aqueduct (12). Notably, Msi1 is expressed in the ependymal cells of both the SCO and the cerebral aqueduct (Fig.

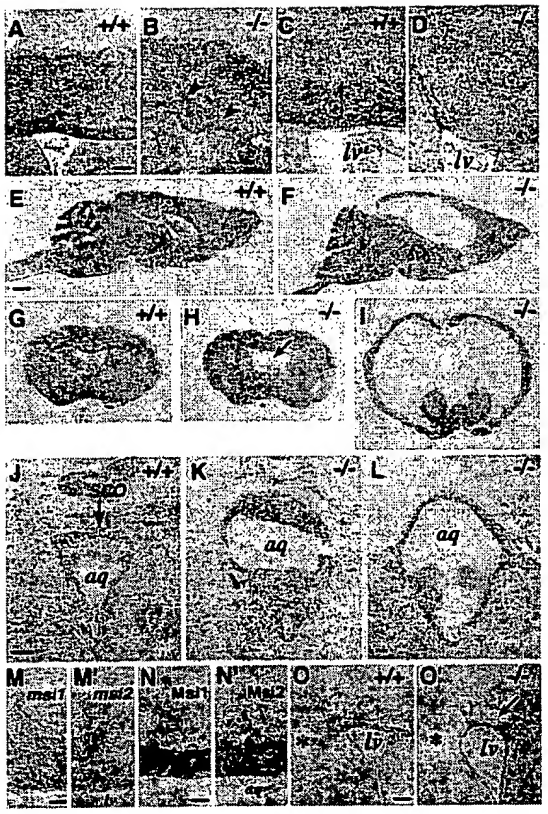

Fig. 2. Development of hydrocephalus in postnatal *msi1*$^{-/-}$ mice with ependymal abnormalities and the concurrent expression of Msi1 and Msi2 in the developing CNS and aqueduct. Genotypes are indicated. (*A–I*) Hematoxylin & eosin-stained adult brain. Agenesis of the CC observed in an adult *msi1*$^{-/-}$ mouse (*B*, arrowheads) compared with the normal CC in a WT littermate (*A*). (*C* and *D*) A higher magnification view of the normal CC and the Probst's bundle, respectively. (*E–I*) Hydrocephalus of the adult *msi1*$^{-/-}$ mice. (*E* and *F*) Sagittal sections. (*G–I*) Coronal sections at the level of the anterior commissure. (*F* and *I*) A severe hydrocephalic mutant showing massive ventricular dilation. (*H*) Moderate dilation of the lateral ventricles accompanied by cavitation of the septum pellucidum and hypoplasia of the septum. (*J–L*) Coronal sections through the aqueduct of the P7 brain, showing abnormal accumulation and polyposis of ependymal cells surrounding the Sylvius aqueduct and SCO. (*M* and *M'*) mRNA *in situ* hybridization analysis of *msi1* and *msi2* in the WT E12.5 telencephalon showing their expression in the VZ. (*N* and *N'*) Immunohistochemical detection of Msi1 and Msi2 in ependymal cells surrounding the aqueduct of WT P3 mice. Immunoreactivities were visualized by DAB (brown), and the nuclei were counterstained with hematoxylin. (*O* and *O'*) Expression of Msi2 in the SVZ and ependymal cells lining the lateral ventricle of a hydrocephalic *msi1*$^{-/-}$ (*O'*) and a WT littermate (*O*) at P3. The *msi1*$^{-/-}$ tissue shows partial destruction of the SVZ (arrow). *lv*, lateral ventricle; *aq*, Sylvius aqueduct; •, septum pellucidum. (Scale bars: *A* and *B*, 150 μm; *C*, *D*, and *J–L*, 50 μm; *E–I*, 1 mm; *M*, 30 μm; *N*, 10 μm; *O*, 100 μm.)

2*N*). Electron microscopic analysis showed an abnormal stratification of the ependymal cells of the *msi1*$^{-/-}$ aqueduct that was not evident in heterozygous or WT littermates. Detailed histological analysis demonstrated that the aqueduct of Msi1 null mice was enwrapped by a thick epithelium composed of two or three layers of ependymal cells, in contrast to the well organized single ependymal cell layer in the aqueduct of WT or heterozygous littermates (Fig. 3*D*). Although the majority of these *msi1*$^{-/-}$ cells appeared to be terminally differentiated ependymal cells, characterized by cilia (Fig. 3 *D* and *F*), some appeared to proliferate heterotopically without nuclear heteromorphism (Fig. 3*E*), which is consistent with the ependymal polyposis in the

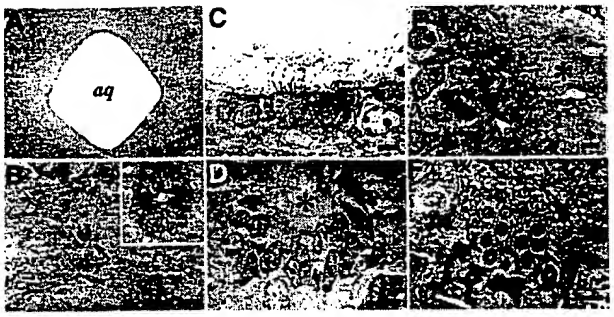

Fig. 3. Light microscopic and ultrastructural analyses of the *msi1*$^{-/-}$ ependyma at P14. (*A* and *B*) Light micrographs of the *msi1*$^{-/-}$ brain showing stenosis of the Sylvius aqueduct (*B*, arrow) and of the WT brain (*A*). *Inset* in *B* shows a low-power view of the ependymal cells in the aqueduct. (*C*) An electron micrograph of the WT aqueduct showing a single layer of ependymal cells and well organized microvilli protruding into the aqueductal lumen. (*D*) The aqueduct of an *msi1*$^{-/-}$ mouse, surrounded by the stratified ependymal epithelium composed of two or three cell layers. (*E*) A high-power view of *msi1*$^{-/-}$ ependymal cells, showing heterotopic mitotic figures without nuclear heteromorphism (arrowhead). Cytoplasmic organelles in these cells appear intact. The lumen of the *msi1*$^{-/-}$ aqueduct is completely filled with amorphous materials (asterisks in *D* and *E*). (*F*) A magnified view of *msi1*$^{-/-}$ ependymal evaginations and cilia. Cilia, characterized by central and peripheral tubules (arrows), are apposed to and are disarranged by solid materials (dashed line) that are abundant in the electron-dense particles of glycogen granules (arrowheads). (Scale bars: *A* and *B*, 40 μm; *B Inset*, 8 μm; *C* and *D*, 5 μm; *E*, 2 μm; *F*, 0.5 μm.)

cerebral aqueduct and SCO. The lumen of the *msi1*$^{-/-}$ aqueduct was completely filled with amorphous materials abundant in electron-dense glycogen granules, and the microvilli of ependymal cells were irregularly arranged, as if they were compressed by the packed materials (Fig. 3*F*).

Hydrocephalus could result from impaired CSF absorption from the subarachnoid space or from overproduction of CSF by the choroid plexus; however, histological examination failed to demonstrate abnormalities in these areas (not shown). Taking these observations together, it is reasonable to attribute the hydrocephalus of *msi1*$^{-/-}$ mice to the obstruction of SCF flow in the aqueduct.

Characterization of CNS Stem Cells Lacking Msi1. We next examined the role of Msi1 in the CNS stem cell population using a neurosphere assay, which is a selective culture system for CNS stem cells (7, 13). In the presence of mitogens such as epidermal growth factor and fibroblast growth factor 2, each CNS stem cell proliferates to form a floating multicellular structure, the neurosphere. Thus, CNS stem cells can be defined as sphere-forming cells (SFCs) (13). The number and size of the formed neurospheres are believed to reflect the number and proliferative activity of SFCs in the dissociated cell population, respectively (neurosphere formation assay). The multipotency of SFCs, that is, their ability to generate different neural cell types, can be assessed by a neurosphere differentiation assay; each neurosphere can be induced to differentiate into TuJ1$^+$ neurons (N), GFAP$^+$ astrocytes (A), and/or O4$^+$ oligodendrocytes (O), after being plated onto substrates in a culture medium containing serum. Consistent with the histological analysis of the *msi1*$^{-/-}$ embryos, the number and size of the neurospheres derived from the *msi1*$^{-/-}$ embryos were quite similar to those derived from WT littermate embryos (Fig. 4 *A* and *D*). When the *msi1*$^{-/-}$ neurospheres were dissociated into single cells, a subset of the cells reformed neurospheres repeatedly, for as many passages as dissociated cells from the WT neurospheres did (not shown). These observations confirmed that the characteristics of the

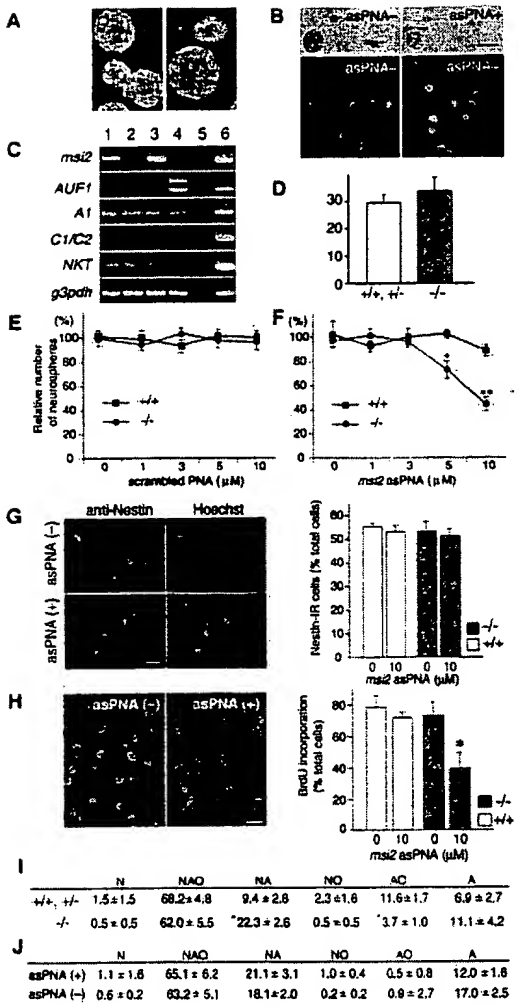

Fig. 4. Loss of *msi1* and *msi2* activities decreased the efficiency of neurosphere formation through the reduced proliferation of SFCs. (*A*) Neurospheres derived from the E14.5 telencephalons of *msi1*$^{-/-}$ or WT littermates (after three passages). (Scale bar, 100 μm.) (*B*) Suppression of Msi2 immunoreactivity on the asPNA-treated SFCs. Dissociated cells from the WT (*Upper*) or *msi1*$^{-/-}$ (*Lower*) primary neurospheres were incubated with or without asPNA (10 μM) for 24 h and immunostained with anti-Msi2 antibody. Immunoreactivities to Msi2 were visualized by a DAB reaction (brown, *Upper*) or Alaxa-568 (red, *Lower*). *Insets* are magnified views of representative cells, showing the repression of Msi2 protein expression without pyknotic changes. [Scale bar, 5 μm (*Inset*) or 25 μm (*Upper* and *Lower*).] (*C*) Semiquantitative RT-PCR analysis of *msi2* mRNA, the *msi*-related genes encoding RNA-binding protein (*AUF1*, *hnRNP A1*, and *hnRNP C1/C2*), *NKT* mRNA, and an internal control mRNA (*g3pdh*). The *msi1*$^{-/-}$ or WT SFCs were incubated with or without asPNA for 24 h, then subjected to the RT-PCR. Lane 1, WT cells; lane 2, WT cells + asPNA; lane 3, *msi1*$^{-/-}$ cells; lane 4, *msi1*$^{-/-}$ cells + asPNA; lane 5, no RT control; lane 6, E14.5 WT cerebral cortex. (*D*) The number of neurospheres per field (×4) formed by the dissociated cells from *msi1*$^{-/-}$ (−/−, $n = 3$) or littermates (+/+, +/−, $n = 3$). (*E* and *F*) The number of neurospheres derived from the telencephalons of *msi1*$^{-/-}$ and their WT littermates in the presence of the scrambled PNA (*E*) or in the presence of the asPNA (*F*). The data were presented as the mean ± SEM (WT, $n = \sim 3$–6; *msi1*$^{-/-}$, $n = \sim 3$–7). $^{*}P < 0.005$, $^{**}P < 0.0001$, in comparison with the WT control (unpaired *t* test). (*G*) The number of nestin^{+} cells after the exposure to asPNA for 24 h. Dissociated cells of the primary neurospheres derived from the WT and *msi1*$^{-/-}$ telencephalons were incubated with or without asPNA (10 μM) and then immunopositive cells for anti-nestin were counted (WT, $n = 6$; *msi1*$^{-/-}$, $n = 6$). Photomicrographs showed the *msi1*$^{-/-}$ cells that were positive for nestin (green). (Scale bar, 25 μm.) (*H*) Decreased number of BrdU^{+} cells within the *msi1*$^{-/-}$ neurospheres exposed to asPNA. During the formation of neurospheres in the presence (+) or absence (−) of asPNA (10 μM), BrdU was administrated at 2 div. After the additional cultivation for 24 h, each sphere was dissociated and immunostained with anti-BrdU (WT, $n = 6$; *msi1*$^{-/-}$, $n = 6$). Photomicrographs represented the BrdU-labeled *msi1*$^{-/-}$ cells (green). The data were presented as the mean ± SEM. *, $P < 0.001$ in comparison with the other conditions (two-tailed Student's *t* test). (Scale bar, 10 μm.) (*I*) Differentiation assay of neurospheres that derived from the *msi1*$^{-/-}$ (−/−) and their littermate cells (+/+, +/−). The differentiation capacity of each primary neurosphere was determined based on the cell types contained in each clone. N, neurons; A, astrocytes; O, oligodendrocytes. The majority (84.5%) of neurospheres from *msi1*$^{-/-}$ mice generated both neurons and glia (NAO+NA+NO). The clone types were analyzed for 98 spheres from WT or heterozygous mice ($n = 5$) and 234 spheres from *msi1*$^{-/-}$ mice ($n = 10$) and presented as the mean ± SEM. *, $P < 0.0001$, in comparison with WT and heterozygous controls (Student's *t* test). (*J*) Differentiation assay of neurospheres that derived from the *msi1*$^{-/-}$ cells treated with or without asPNA. The clone types were analyzed for 67 spheres from asPNA (+) and 141 spheres from asPNA (−).

msi1$^{-/-}$ stem cells *per se* were unchanged; disruption of the *msi1* gene alone did not affect the number or self-renewal activity of CNS stem cells.

A clonogenic differentiation assay using cell-type-specific markers revealed that the majority of SFCs from *msi1*$^{-/-}$ mice were multipotent and could give rise to all three types of differentiated cells (NAO) (Fig. 4*I*). Nonetheless, we noticed that the *msi1*$^{-/-}$ SFCs showed a slightly limited repertoire for differentiation: the number of clones containing oligodendrocytes (AO+NO+NAO) tended to be slightly smaller in the *msi1*$^{-/-}$ neurospheres (66.2%) than in the WT neurospheres (82.1%). This reduction could not be restored by supplementing the differentiation medium with retinoic acid or T3, agents that potentiate the differentiation and proliferation of oligodendrocyte precursors (not shown). Thus, a lack of *msi1* activity reduces the multipotency of the CNS stem cells.

The Msi Family Functions in the Proliferation of Neural Progenitor Cells Including CNS Stem Cells. Taking into account the high expression levels of *msi1* during embryonic development (2), it was somewhat surprising that *msi1*$^{-/-}$ mice survived embryonic development with just minor structural abnormalities in restricted populations of ependymal cells. This finding indicates that *msi1* is not essential for the development of most tissues and body structures. In addition, a small fraction of *msi1*$^{-/-}$ mice (5–10%) survived into adulthood without detectable morphological abnormalities. These observations raised the possibility that the loss of *msi1* functions might be compensated for, at least in part, by one or more other genes. One such candidate was *msi2*, another member of the Msi family (4). In the CNS, the expression of Msi2 is developmentally regulated and overlaps with that of Msi1 in neural precursor cells, astrocytes, and ependymal cells (Fig. 2 *M–O*) (4). The Msi2 expression level was up-regulated 1.4–2.0-fold in the *msi1*$^{-/-}$ embryonic brains (E12.5), compared with WT brains, as determined by Western (Fig. 1*C*) and Northern (not shown) blotting. These results support the hypothesis that *msi2* compensates for the *msi1* deficiency during the CNS development of *msi1*$^{-/-}$ mice.

To test directly for the cooperative involvement of Msi family proteins in the function of CNS stem cells, we tried making a functional double knockout of *msi1* and *msi2*. To this end, we added antisense compounds specific to the *msi2* gene to the medium of a CNS stem cell culture prepared from *msi1*$^{-/-}$ embryos or WT littermates and assessed the resulting number of neurospheres. Antisense oligonucleotides covering the translation initiation region or the coding region of *msi2* (a 16-mer) was synthesized as peptide nucleic acids (asPNA). PNA is a new type of DNA analogue that has an artificial homomorphous peptide backbone with much higher sequence specificity for target genes,

a higher resistance to proteases and nucleases, and lower cytotoxicity (14) than other forms of antisense nucleotides, such as the phosphorothioate oligos. Administration of asPNA to cultures derived from the WT or *msi1*$^{-/-}$ embryonic forebrain led to a specific and marked reduction in *msi2* expression at both the mRNA and protein levels, as demonstrated by semiquantitative RT-PCR analysis and immunocytochemical detection with the anti-Msi2 antibody (Fig. 4 *B* and *C*). With the concentrations of the asPNA used in this series of experiments, rapid cellular changes such as nuclear condensation were not observed in most of the individual dissociated cells, confirming that there was no or very little cytotoxicity associated with the asPNA. Homology search with this asPNA sequence against the GenBank/EST or the Celera Discovery System and Celera Genomics' associated databases indicated that any other mRNAs did not contain a completely matched sequence to the designed sequence of the *msi2* asPNA. We performed the control RT-PCR analysis of the organic cation transporter *NKT* (GenBank accession no. U52842), which was expressed in the neurospheres and shared a sequence similarity to asPNA with a two-base mismatch. As shown in Fig. 4*C*, the asPNA treatment had no effect on the level of *NKT* mRNA expression in the *msi1*$^{-/-}$ or littermate cultures, confirming the target specificity of asPNA. In the neurosphere formation assay, the presence of the asPNA caused a drastic reduction in the number of neurospheres formed in the *msi1*$^{-/-}$ cultures in a dose-dependent manner (Fig. 4*F*). In contrast, the greatest concentration of asPNA used in our study did not decrease the number of neurospheres formed in the WT cultures, indicating that the suppression of Msi2 alone did not affect the neurosphere-forming ability and viability of CNS stem cells. Administration of the nonspecific scrambled PNA did not affect the number of neurospheres formed in both the WT and *msi1*$^{-/-}$ cultures (Fig. 4*E*), excluding the possibility that the *msi1*$^{-/-}$ CNS stem cells are more susceptible to PNA-mediated cytotoxicity than WT cells. We, therefore, concluded that the reduction of neurosphere formation mediated by asPNA is a specific effect and that a cooperative action of both Msi1 and Msi2 is essential for the proliferation and/or maintenance of embryonic CNS stem cells.

We sought to establish the characteristics of *msi1*$^{-/-}$ CNS stem cells that exposed to the asPNA. Although there are no unambiguous markers for CNS stem cells, we analyzed the number of cells that express nestin, a marker for neural progenitor cells including CNS stem cells (1). Dissociated cells from the primary neurospheres were incubated with the asPNA during the first 24 h before the majority of the SFCs started cell division. Immunocytochemical analysis indicated that nestin$^+$ cells were ≈55% of total cells in WT and *msi1*$^{-/-}$ cells, and the number of nestin$^+$ cells were unchanged with or without asPNA treatment (Fig. 4*G*), suggesting that the asPNA treatment did not influence the nestin expression and that there were the equivalent number of progenitor cells at the beginning of their sphere formation. Next, we determined the effect of asPNA on the proliferation of neural progenitor cells including CNS stem cells. BrdU was administrated into the sphere cultures that have pretreated with asPNA for 2 div. Twenty-four hours later (3 div), the individual forming neurospheres, which were in a phase of the exponential proliferation of SFCs, were harvested, and the BrdU-labeled cells were counted. Without the asPNA treatment, the labeling index for BrdU was unchanged in *msi1*$^{-/-}$ neurospheres relative to the WT (≈75% of total cells); however, the marked reduction of BrdU-labeled cells was evident in the asPNA-treated *msi1*$^{-/-}$ neurospheres (≈40% of total cells) (Fig. 4*H*). Neurosphere differentiation assay revealed that the asPNA treatment did not lead a significant change in the differentiation ability in the *msi1*$^{-/-}$ SFCs (Fig. 4*J*). Taken together, the reduction of neurosphere formation in *msi1*/*msi2* functional double knockout cells was primarily attributable to the inhibition of the SFC proliferation, rather than due to an aberrant cell-fate choice. Because the short exposure of the asPNA (1 div) seemed to be unaffected for the number of nestin$^+$ cells, *msi1* and *msi2* may not be involved directly in a survival of the neural progenitor cells including CNS stem cells.

Among the numerous RNA-binding proteins, Msi2 and Msi1 form a unique subgroup in the hnRNP A/B class that contains two copies of RRMs. Within this class, the Msi family has a sequence similarity to AUF1 (hnRNP D) and hnRNP A1, albeit to a lesser degree (4). To assess whether the expression levels of these *msi*-related genes altered in the functional double knockout cells, we performed a semiquantitative RT-PCR analysis. A significant up-regulation of the mRNA for AUF1 was detected in the asPNA-treated *msi1*$^{-/-}$ cultures without affecting the expression levels of hnRNP A1 and hnRNP C1/C2 (Fig. 4*C*).

Discussion

Here, we showed that disruption of the *msi1* gene causes hydrocephalus. The abnormal proliferation and/or differentiation of ependymal cells lining the aqueduct may be the primary cause of the obstruction of the CSF passage, which results in the perinatal onset of hydrocephalus in *msi1*$^{-/-}$ mice. However, we found no significant difference in the total number of ependymal cells lining the whole area of the aqueduct in perinatal *msi1*$^{-/-}$ pups, as determined by counting the cells in serial sections (not shown). In addition, the BrdU pulse-labeling experiments at P3 and P10 brains showed that there was no difference in the total number of proliferating cells along the aqueductal ependyma between *msi1*$^{-/-}$ pups and their WT littermates (not shown), in contradiction to the stratification and polyposis formation in the postnatal SCO. These morphologically abnormal ependymal cells were rarely labeled with BrdU at least by 4 h of BrdU labeling at P3 (not shown). They may proliferate slower than other normal ependymal populations during the aqueductal development. Collectively, Msi1 is likely to be essential for the differentiation or proper functions of the highly restricted cell populations in the ependyma lining the aqueduct. Conceivably, these cells may have a higher susceptibility to the deficiency of the *msi1* gene than do the ependymal cells located in other regions of the ventricular system. Nevertheless, considering the coexpression of Msi1 and Msi2 in most ependymal populations (Fig. 2*N*), it remains unclear why the defects in *msi1*$^{-/-}$ mice are restricted to a certain population of ependymal cells. Msi1 may have a specific function that is required for the development of aqueductal ependymal cells.

The CNS stem cells continue to proliferate in an undifferentiated state (self-renewal proliferation), and also can give rise to the lineage-restricted progenitors, i.e., the neuronal or glial progenitors. The antisense ablation experiments of the *msi2* gene indicated the vital functions cooperated with the *msi* genes during the formation of the neurosphere. Functionally double knockout neurosphere cells showed the reduction of proliferation. This finding suggests that two *msi* genes are involved in the self-renewal proliferation of CNS stem cells. Alternatively, such phenomena could have been caused by enhanced differentiation of CNS stem cells to lineage-restricted progenitors. However, the differentiation potential of double knockout cells was not significantly changed from that of *msi1*$^{-/-}$ cells, suggesting that the *msi2* gene is not essential for the lineage restriction of CNS stem cells.

Recent *in vitro* and *in vivo* studies have provided intriguing evidence regarding a source of CNS stem cells in the postnatal CNS. Johansson *et al.* (15) showed that CNS stem cells could originate from ependymal cells in the adult brain and spinal cord, whereas Doetsch *et al.* (16) provided compelling evidence that SVZ astrocytes are precursors for neurogenesis and could possibly serve as stem cells. Although it has been controversial which type of cells serves as the source of postnatal CNS stem

cells *in vivo*, the disorganized proliferation or premature differentiation of *msi1*$^{-/-}$ ependymal cells may reflect an impairment of the proliferation or maintenance of CNS stem cells residing in the aqueductal ependyma. However, this idea regarding the ependymal proliferation caused by the loss of *msi1* seemed to be inconsistent with the proposed function of *msi* genes that positively regulate the proliferation of CNS stem cells. It is possible that there is a unique function of the *msi1* gene that keeps the aqueductal ependymal cells quiescent that the *msi2* gene cannot compensate for. Alternatively, the *msi2* gene may be functionally "overexpressed" in the absence of *msi1* in the restricted aqueductal ependymal cell population. Because the Msi family proteins bind to a similar RNA sequence, excessive binding of Msi2 protein to the target mRNAs normally occupied by the Msi1 protein could account for the ependymal proliferation through the misregulation of target mRNAs. Such ependymal proliferation might be implicated in the genesis of ependymal tumors, ependymomas. In fact, of the various brain tumors, the expression levels of the Msi family proteins are markedly elevated in ependymoma cells (unpublished results). Msi1 expression also correlates well with the proliferative activity and malignancy of human gliomas (6), suggesting the involvement of deregulated Msi1 expression in tumorigenesis. Appropriately and tightly regulated expression levels of the *msi* family genes may be crucial not only for the proper development of neural precursors but also to prevent the clonal expansion of proliferative tumors.

Msi1 and Msi2 show a marked sequence similarity, especially within their RRM domains (≈90%), which are responsible for specific binding with target RNA molecules. Our previous *in vitro* RNA-binding assay (4) revealed that Msi1 and Msi2 have a very similar RNA-binding specificity characterized by uridine-rich sequences. The cooperative and redundant functions of *msi2* and *msi1* in CNS stem cells further support the idea that Msi1 and Msi2 interact with common target RNAs *in vivo*. In addition, our present RT-PCR analysis indicated the up-regulation of AUF1 mRNA in the asPNA-treated *msi1*$^{-/-}$ cultures. AUF1 is a key regulatory factor of gene expression that is known to bind uridine-rich elements of 3′-UTRs and regulate the stability of many protooncogene and cytokine mRNAs (17). It is unclear whether the Msi proteins and AUF1 share the common downstream target mRNAs. Therefore, we could not exclude a possibility that the loss of two *msi* gene activities could have been compensated by the enhanced expression of AUF1 in the neurospheres derived from the *msi2* asPNA-treated *msi1*$^{-/-}$ cells (Fig. 4*F*). The molecular mechanism underlying the functions of the Msi family should be elucidated by identifying their target mRNAs. One of the candidates is the mRNA of mammalian *numb* (*m-numb*), which encodes a membrane-associated antagonist of Notch signaling (18, 19). The Notch signal is required for the self-renewing activity of mammalian CNS stem cells (7, 20, 21). Our recent study (22) indicated that Msi1 binds to the uridine-rich sequence in the 3′-UTR region of *m-numb* mRNA *in vitro* and can repress the expression of *m-numb* at the level of translation in NIH 3T3 cells. These results, combined with the observation that overexpressed Msi1 activates Notch signaling (22), indicate that the Msi family may contribute to the self-renewing activity of CNS stem cells by modulating the *m-numb*-Notch signaling cascade. However, our immunohistochemical study with an anti-*m*-Numb antibody failed to demonstrate a significant increase in the *m*-Numb protein in the neurospheres or brain of *msi1*$^{-/-}$ mice (not shown). Considering that the up-regulated *msi2* acts as a redundant gene in the *msi1*$^{-/-}$ CNS, loss-of-function experiments with both the *msi2* and *msi1* genes may unequivocally reveal the function of the Msi proteins in mammalian CNS development. We have generated a homozygous mutant harboring a disrupted *msi2* allele, and the phenotypic analyses of double-knockout mice with an *msi2/msi1* genotype are currently in progress in our laboratory.

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Nestin-EGFP Transgenic Mice: Visualization of the Self-Renewal and Multipotency of CNS Stem Cells

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We generated transgenic mice carrying enhanced green fluorescent protein (EGFP) under the control of the *nestin* second-intronic enhancer (E/nestin:EGFP). Flow cytometry followed by *in vitro* assays revealed that *in situ* EGFP expression in the embryonic brain correlated with the mitotic index, the cogeneration of both neurons and glia, and the frequency of neurosphere formation *in vitro*. High-level EGFP expressors derived from embryos included a distinct subpopulation of cells that were self-renewable and multipotent, criteria that define neural stem cells (NSCs). Such cells were largely absent among lower-level or non-EGFP expressors, thereby permitting us to enrich for NSCs using EGFP expression level. In adults, although E/nestin:EGFP-positive cells included the NSC population, the frequency of neurosphere formation did not correlate directly with the level of EGFP expression. However, moderately EGFP-expressing cells in adults gained EGFP intensity when they formed neurospheres, suggesting embryonic and adult NSCs exist in different microenvironments *in vivo*.

INTRODUCTION

During mammalian development, stem cells play a critical role in histogenesis and organogenesis, and in the maintenance of the resulting structures by providing the phenotypically restricted progenitors from which differentiated phenotypes arise. The biology of stem cells, especially the molecular mechanisms that regulate their generation, maintenance, and differentiation, is best studied using methods in which the behaviors of isolated or enriched populations of stem cells can be analyzed under carefully controlled conditions (Morrison *et al.*, 1997, 1999; Barres, 1999). For such studies, the ability to perform "prospective identification," that is, to identify live stem cells in dissociated native tissue, is crucial. In the central nervous system (CNS), however, the lack of surface antigens specific to neural stem cells (NSCs) prohibits applying cell sorting techniques to the study of NSCs; as a result, NSCs have not yet been readily identifiable from mammalian brain tissue. Intriguingly, Johansson *et al.* (1999) found that ependymal cells in the adult brain can be isolated based on Notch1 expression, and that these cells exhibit the cellular properties of stem cells *in vitro*. Doetsch (1999) used different methodologies to conclude that at least some subependymal astrocytes may be adult NSCs. Yet although provocative, neither of these studies involved the prospective identification of NSCs. The need to further understand these intriguing results has led in turn to a critical need for a means to locate, identify, and select live CNS stem cells.

To achieve this end, we used green fluorescent pro-

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tein (GFP), placed under the transcriptional control of the neural-specific second intronic enhancer of the *nestin* gene (Zimmerman *et al.*, 1994), as a live-cell reporter of the neural progenitor phenotype. Nestin is an intermediate filament transiently expressed during neural ontogeny; in development, it is expressed first by neuroepithelial cells and radial glia, and later by progenitor cells of the ventricular zone (during the embryonic stage) and the nascent ependyma/subependyma (during the postnatal stage) (Hockfield and McKay, 1985; Frederiksen and McKay, 1988; Lendahl *et al.*, 1990; Doetsch *et al.*, 1997; Lothian and Lendahl, 1997). Using a variety of neural cell markers, we recently found that a subset of embryonic brain cells shows a particularly high level of immunoreactivity for Nestin, as well as for the neural RNA binding protein, Musashi-1 (Kaneko *et al.*, 2000). This observation suggested that Nestin might be expressed at different levels among different classes of CNS progenitor cells, despite its seemingly ubiquitous expression by the embryonic neuroepithelium. Furthermore, among the marker molecules reported to label CNS progenitor cells, only the *nestin* gene has a cis-element, within its second intron, that is well characterized and available for expressing foreign genes *in vitro* and *in vivo* (Zimmerman *et al.*, 1994; Lothian and Lendahl, 1997).

Based on these facts, we postulated that if we could drive GFP expression using the cis regulatory elements for *nestin*, we could fractionate progenitor cells based on the intensity of the GFP fluorescence. We have already

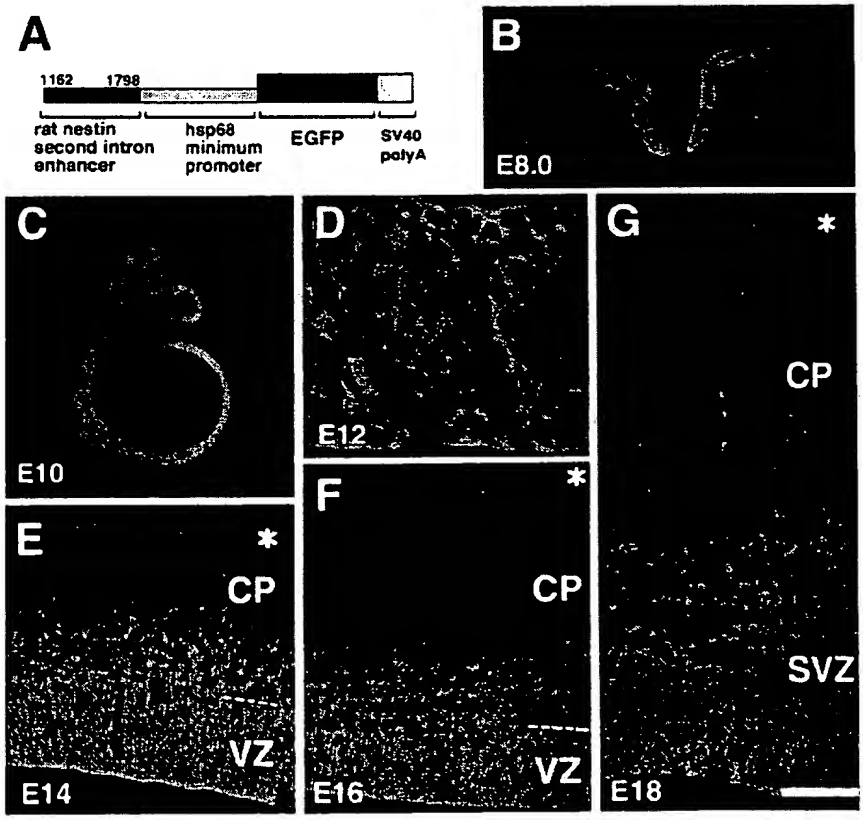

FIG. 1. E/nestin:EGFP-transgenic mice. (A) Structure of the E/nestin:EGFP transgene. The second intron enhancer of the rat *nestin* gene (1162–1798) (Lothian and Lendahl, 1997) was placed upstream of the minimum promoter of heat shock protein 68 (hsp68) (Rossant *et al.*, 1991) fused to EGFP cDNA and a polyadenylation signal. (B) Section of an E8.0 embryo, showing EGFP expression restricted to the neural plate. (C) Whole-mount fluorescent micrograph showing EGFP expression throughout the CNS of an E10 embryo. (D–G) Section of the cerebral wall at E12 (D), E14 (E), E16 (F), and E18 (G). At E12, EGFP fluorescence was detected from the ventricular to the pial surface of the cerebral wall, but the overall level of EGFP expression was lower than at E14–18. At E14–18, EGFP was predominantly expressed in the ventricular zone (VZ), and not in the cortical plate (CP). Asterisk: pial surface. CP, cortical plate; VZ, ventricular zone; SVZ, subventricular zone. Bar: 80 μm in B; 1.3 mm in C; 27 μm in D; 90 μm in E–G.

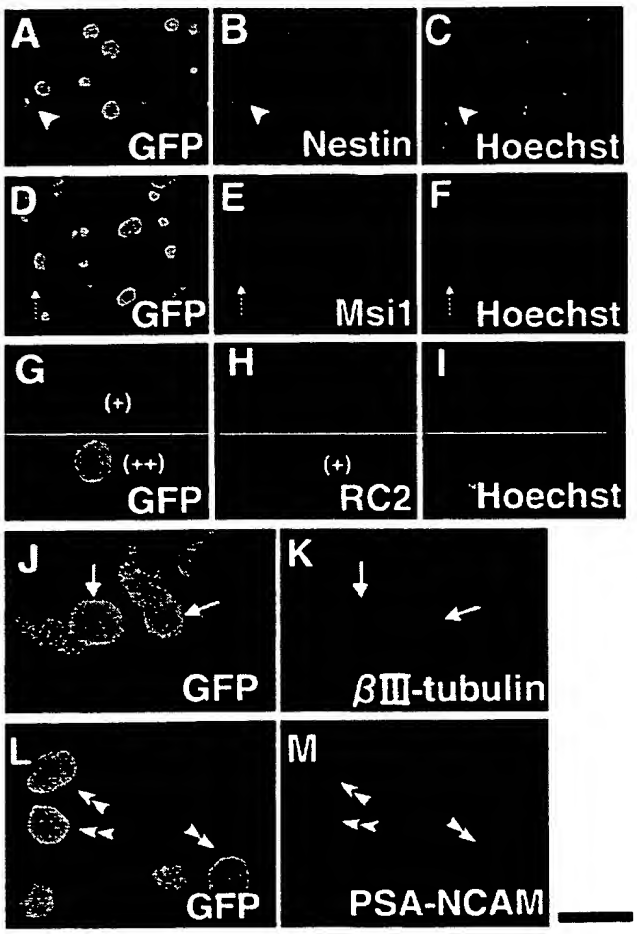

FIG. 2. Immunocytochemical characterization of EGFP-expressing cells. Forebrain cells harvested from E14-15 E/nestin:EGFP mice were doubly immunostained with anti-GFP (A, D, G, J, L) and anti-Nestin (Rat401) (B), anti-Musashi1 (E), RC2 (H), anti-βIII-tubulin (K), or anti-PSA-NCAM (M) antibodies. Both $EGFP^+$ cells (arrowhead in A) and $EGFP^{2-}$ cells were $Nestin^+$ (B). Musahsi1 immunoreactivity was detected in $EGFP^{2+}$ cells, but also in some $EGFP^-$ cells (dashed arrow in D-F). RC2 immunoreactivity was almost completely confined to $EGFP^{2-}$ cells (G, H). βIII-tubulin was detected in some $EGFP^+$ cells but in no $EGFP^{2+}$ cells (arrows, J, K). $EGFP^{2+}$ cells were also PSA-NCAM negative (double-arrowheads, L, M). Bar, 50 μm in A-F, 20 μm in G-M.

used *nestin* second intronic enhancer-driven EGFP (E/nestin:EGFP) to identify neuronal or neural progenitor cells in both the adult human periventricular area and hippocampus (Roy *et al.*, 2000a,b). However, these experiments were performed by transfecting dissociated brain cells with E/nestin:EGFP plasmid DNA. As a result, the intensity of GFP-fluorescence, and hence the yield of identified progenitor cells, likely varied as a function not only of the transfection efficiency (9.40 ± 0.9%, mean ± SEM) (Roy *et al.*, 2000b), but also of the plasmid copy number in each successful transfectant. In addition, the transient nature of plasmid transfection precluded the use of this technique for the long-term observation and monitoring of sorted progenitor cells, whether *in vivo* or *in vitro*.

To address these concerns, we took a transgenic approach towards fractionating neural progenitor cells on the basis of *nestin* transcription. To this end, we established transgenic mice in which *nestin* enhancer-driven transcriptional activation can be reported by EGFP expression. In the embryonic brains of these transgenic mice, cells expressing a high level of EGFP are largely limited to the ventricular zone (VZ). Using flow cytometry combined with the *in vitro* examination of clonal or neurosphere cultures of sorted cells, we found that the activation of the *nestin* enhancer is highest in cells that can potentially proliferate, self-renew, and generate both neurons and glia, which are all criteria that define NSCs. Thus, this mouse line provides a useful tool with which to monitor these developmental characteristics in culture and assess them *in vivo*.

RESULTS

EGFP Expression was Restricted to the Ventricular Zone in E/nestin:EGFP-Transgenic Mice

Transgenic mice expressing EGFP under the control of the rat *nestin* second intronic enhancer were generated (Fig. 1A). We obtained six independent lines of E/nestin:EGFP transgenic mice that showed indistinguishable patterns of EGFP expression in the developing CNS, indicating the consistent expression profile of this transgene (Fig. 1A).

By microscopic examination, the EGFP expression was detectable as early as E7. At this stage, the anti-GFP immunoreactive cells were sporadically distributed in the one-cell-thick neural ectoderm (data not shown). At E8, EGFP expression was readily detected in the neural plate (Fig. 1B). At E10, EGFP fluorescence was seen throughout the CNS (Fig. 1C).

We further examined the stage-dependent changes in the expression pattern of EGFP, focusing on the cerebral wall where the distribution of proliferative and differentiated cells has been extensively studied (Smart, 1983; Takahashi *et al.*, 1995). At E12 (Fig. 1D), EGFP expression was detected in the ventricular zone (VZ), but both the overall and highest expression levels were lower than at E14 (Fig. 1E) and later (Figs. 1F and 1G). During

the period of active neuronogenesis and formation of the cortical plate (CP) (Figs. 1E and 1F), a high level of EGFP expression was seen in the ventricular side containing the VZ, and the fluorescence was strongest along the ventricular surface. At E18, EGFP expression in the VZ had become sparse, but was still highest along the ventricular side (Fig. 1G).

Strongly E/nestin:EGFP-Positive Cells Expressed Progenitor Markers, But No Mature Neural Markers

We next examined the antigenic phenotypes of brain cells categorized on the basis of their E/nestin:EGFP expression. Microscopic observation of cerebral cells dissociated from E14–15 mice revealed three categories of cells with respect to their EGFP levels: negative ($EGFP^-$, a level indistinguishable from that of cells derived from wild-type mice), weakly positive ($EGFP^+$), and strongly positive ($EGFP^{2+}$). $EGFP^{2+}$ cells were three to five times brighter than $EGFP^+$ cells when measured by Photoshop (Adobe Systems). About 25% of the total cerebral wall cells at E14 were $EGFP^{2+}$.

All E/nestin:EGFP-expressing cells (both $EGFP^{2+}$ and $EGFP^+$) expressed Nestin (Figs. 2A–2C), and Musashi-1, another antigenic marker of neural progenitor cells, including NSCs (Sakakibara *et al.*, 1996; Kaneko *et al.*, 2000) (Figs. 2D–2F) (Table 1). Immunoreactivity for RC2, which is selectively expressed by radial glia and neural progenitor cells (Misson *et al.*, 1988; Kaneko *et al.*, 2000), was detected in $EGFP^{2+}$ cells, of which about 70% were $RC2^+$ (Figs. 2G–2I) (Table 1). Conversely, $RC2^+$ cells almost invariably expressed both E/nestin:EGFP fluorescence and Musashi-1 immunoreactivity. Thus, RC2-defined radial cells comprised a subset of E/nestin:EGFP-positive ventricular zone cells.

In general, E/nestin-driven EGFP expression was more robust in undifferentiated progenitor cells than in their differentiated daughters: $EGFP^{2+}$ cells failed to express neuronal βIII-tubulin (Figs. 2J and 2K) or MAP2 (Table 1), astroglial GFAP, or oligodendroglial O4 (not shown). The $EGFP^{2+}$ cells also failed to express detectable PSA-NCAM (Seki and Arai, 1991) (Figs. 2L and 2M) (Table 1). Notably, some $EGFP^+$ cells were βIII-tubulin-positive (Figs. 2J and 2K), suggesting that phenotypic maturation was accompanied by down-regulation of the E/nestin:EGFP expression.

Time-Lapse Observation of the Cytogenesis of Embryonic $EGFP^{2+}$ Cells

We further assessed the lineage potentials of $EGFP^{2+}$ cells *in vitro*. First, cerebral cells dissociated from E14 transgenic embryos were plated onto polyethylenimine (PEI)-coated plastic dishes at a low density, and $EGFP^{2+}$ cells ($n > 50$) were chosen upon the initial microscopic observation (<2 h after plating). The behavior of each chosen cell was then time-lapse recorded.

About 50% of the total $EGFP^{2+}$ cells observed divided within 24 h, and the remaining $EGFP^{2+}$ cells died. Figure 3A shows the generation of two daughter cells from a single $EGFP^{2+}$ cell, in which one of the daughter cells expressed a lower level of EGFP (indicated by a single arrowhead) and was βIII-tubulin$^+$, while the other retained a high level of EGFP expression (double-arrowheads in Fig. 3A) and was βIII-tubulin negative. Clones generated by single $EGFP^{2+}$ cells by 1 day in culture often (about 50%) contained cells that were different, both in their morphology and expression of molecular markers.

In longer (>3 day) culture, most (60–80%) clones that were formed by single $EGFP^{2+}$ cells contained both neurons (βIII-tubulin$^+$) and astrocytes ($GFAP^+$) that did not show (or only faintly showed) EGFP fluorescence (Fig. 3B). These observations indicate that (1) the intensity of EGFP fluorescence may reflect cells' developmental status or potential, and that (2) $EGFP^{2+}$ cells can divide to generate both neurons and glia *in vitro*.

To further assess the ability of the EGFP-expressing cells to continue to proliferate, we used neurosphere cultures (Reynolds and Weiss, 1992, 1996). In the presence of EGF and/or bFGF, CNS stem cells proliferate in a self-renewing manner to generate cell aggregates, or spheres, containing mostly undifferentiated cells. Time-lapse recording of sphere formation from single $EGFP^{2+}$ cells (Fig. 4A) demonstrated that newly generated cells showed EGFP fluorescence at a level similar to that of

TABLE 1
Antigen Expression of $EGFP^{2+}$ Cells from E14 Embryos

α-Nestin	100%
α-Musashi1	100%
RC2	69.2 ± 8.6%
α-βIII-tubulin	0%
α-MAP2	0%
α-PSA-NCAM	0%

Note. These cells expressed progenitor but not neuronal markers. Data indicate the mean ± SD from 3 samples (>200 $EGFP^{2+}$ cells in each sample).

the sphere-initiating cells during the first 2–3 days, until the sphere became too large for analysis at the single-cell level. By 7 days, highly fluorescent spheres had formed. More than 90% of the cells within these "bright" spheres coexpressed E/nestin-driven EGFP ($EGFP^+$ or $EGFP^{2+}$) and Nestin protein (*not shown*). Of the total cells dissociated from these spheres, 50–70% were $EGFP^{2+}$. Interestingly, $RC2^+$ immunoreactivity was also detected in 50-70% of the dissociated cells (not shown), consistent with our previous report that Musashi-1^+/$Nestin^+$/$RC2^+$ cells comprised the largest antigenic phenotype in spheres (Kaneko *et al.*, 2000). Since the proportion of $EGFP^{2+}$ cells in the embryonic cerebral cells before culturing was about 25% *in vivo* at E14, the sphere culture resulted in a greater enrichment in the $EGFP^{2+}$ cell population (50–70%).

Bright spheres transferred onto PEI-coated dishes formed cellular sheets within several days (Fig. 4B). At the thinnest (most well spread) part of the bright sphere-derived sheets, EGFP fluorescence was lost. In these areas, βIII-tubulin$^+$ neurons, $GFAP^+$ astrocytes, and $O4^+$ oligodendrocytes were observed (Fig. 4C). This result indicates that the sphere-initiating $EGFP^{2+}$ cells were multipotent.

Although the intensity of EGFP fluorescence in monolayer cultured cells seemed to be affected to some degree by cell shape (Fig 3B), these *in vitro* results overall suggest that the EGFP expression levels correlate with the developmental potentials, as well as the antigenic phenotypes, of E14 cerebral cells *in vitro*. Therefore, this transgenic mouse line may be useful to infer in real time these important aspects of cellular functions during development.

E/nestin:EGFP-Based FACS Yields a Population of $Nestin^+$/βIII-$Tubulin^-$ Cells

The preceding observations suggested to us that as a population, E/nestin:EGFP-positive cells included multipotential neural progenitor cells. To determine directly the correlation between EGFP expression and cell properties, and whether cells displaying the highest level of EGFP fluorescence fulfill the definitional criteria for NSCs, we subjected embryonic E/nestin:EGFP-transgenic brain cells to fluorescence-activated cell sorting (FACS).

Dissociated E14 murine brain cells were sorted into one of three fractions, each defined by its EGFP-fluorescence intensity as measured by the FL1 photomultiplier of the cell sorter (Fig. 5). These groups were defined so that the numbers of cells gated into the three fractions (F^-, F^+, and F^{2+}; Fig. 5) were 30, 60, and 10% of the total number of cells applied, respectively. The proportions of sorted cells that were optically judged as $EGFP^{2+}$ (determined by live examination and immunochemically) were >95% in F^{2+}, 20–25% in F^+, and <1% in F^-.

First, we characterized the cells in these three fractions using the antibodies against Nestin protein and βIII-tubulin. As expected, $Nestin^+$ cells were most abundant in F^{2+} with a proportion of 98.2 ± 0.3% (mean ± SD), and the proportions of $Nestin^+$ cells were lower in F^+ and F^- (68.0 ± 15.7% and 24.9 ± 14.4%, respectively) (Table 2A). The level of Nestin immunoreactivity per positive cell also tended to be lower in the F^-group. The proportions of βIII-$tubulin^+$ cells showed a reciprocal pattern to those of $Nestin^+$ cells: 0.9 ± 0.3% in F^{2+}, 37.5 ± 7.7% in F^+, and 69.8 ± 12.0% in F^- (Table 2A). Both Nestin and βIII-tubulin were simultaneously expressed in some cells sorted into F^+ (20–30%) and F^- (10–20%), probably reflecting a transition in the expression of these proteins during neuronal differentiation (Frederiksen and McKay, 1988; Kaneko *et al.*, 2000). These data indicated that E/nestin-driven EGFP fluorescence intensity correlated with Nestin protein expression and that both were negatively associated with neuronal antigenic maturation.

E/nestin:$EGFP^{2+}$ Ventricular Zone Cells Are Mitotic

To assess the mitotic competence of those cells having the highest level of EGFP-fluorescence, we next determined whether they were proliferating *in vivo*. Forebrain cells were isolated from E14 embryos whose mothers had been given BrdU twice during pregnancy (40 and 10 min before hysterotomy). The cells were then sorted by FACS, plated, and immunostained shortly (<1 h) thereafter. The resulting BrdU-labeling indices (Table 2B) suggested that those cells exhibiting the strongest transcriptional activation of the *nestin* enhancer were the most likely to be at S phase as a population at this embryonic stage.

We next examined whether E/nestin:$EGFP^{2+}$ cells were mitotic *in vitro* as well as *in vivo*. Sorted E15 forebrain cells were plated onto PEI-coated coverslips, cultured 12 h in DMEM/F12-based serum-free media with EGF and bFGF (20 ng/ml each), then treated for 1 h with BrdU (10 μM), fixed, and stained with anti-BrdU. The BrdU-labeling index of the F^{2+} cell fraction (33.7%) contrasted sharply with that of F^- cells (3.1%) (Table 2B), indicating that, under these culture conditions, cells with the strongest EGFP fluorescence were selectively mitotic relative to fetal brain cells in which *nestin* transcription was no longer active.

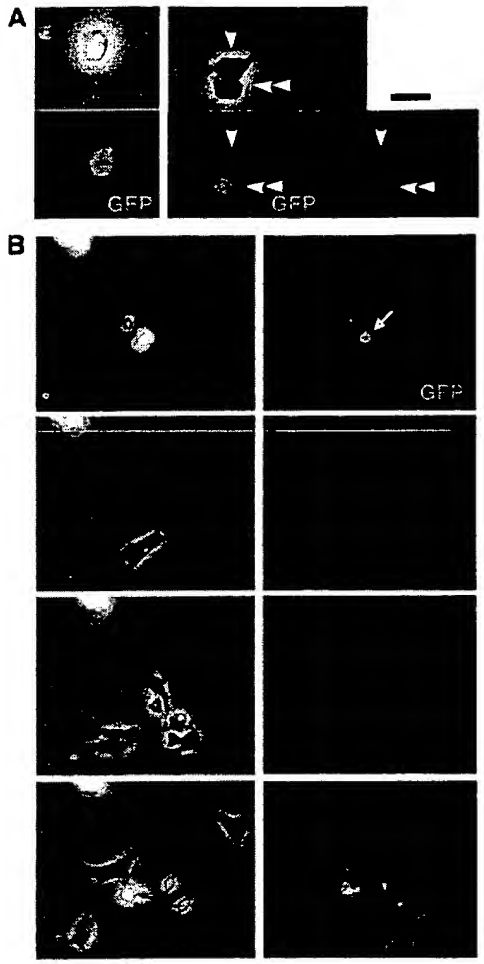

FIG. 3. Developmental potentials of embryonic $EGFP^{2+}$ cells in monolayer culture. Time-lapse observation of the differentiation of a single $EGFP^{2+}$ cell of the E14 cerebral wall in monolayer culture. The number in each pair of phase-contrast and fluorescent pictures indicates the day in culture. (A) A single $EGFP^{2+}$ cell divided by 1 day to produce two daughter cells showing different levels of EGFP fluorescence. One of them expressed a lower level of EGFP fluorescence (arrowhead) and was positive for the neuronal marker βIII-tubulin$^+$, while the other cell expressed a higher level of EGFP (double-arrowheads) and was βIII-tubulin$^-$, indicating that this cell division was asymmetric. (B) A single $EGFP^{2+}$ cell (arrow) produced neurons and astrocytes in the monolayer culture. During differentiation of the cell, EGFP fluorescence intensity decreased. By day 3, the EGFP fluorescence had decreased to a nondetectable level, and the clone consisted of βIII-tubulin$^+$ neurons (green) and $GFAP^+$ astrocytes (red). Bar: 20 μm in A, 50 μm in B.

$EGFP^{2+}$ Cells Generate Neurons and Astrocytes in Low-Density Monolayer Culture

To assess the lineage potential of the sorted cells from the E14 forebrain, we cultured them on PEI-coated plastic dishes at a low density (750 cells/cm^2) for 7 days. Clones formed by these cells were categorized into three groups according to immunocytochemical results (Table 2C): (1) clones consisting of βIII-tubulin$^+$ neurons only ("N" clones), (2) clones having $GFAP^+$ astrocytes but not neurons ("A" clones), and (3) clones having both neurons and astrocytes ("N + A" clones). "N + A" clones were found in F^-, F^+, and F^{2+} cultures; however, the frequency of their formation was highest in the F^{2+} (79.2%, n = 221 clones), compared with F^+ (30.0%, n = 135), and F^- (15.5%, n = 71) (Table 2C). However, these values were affected by differences in plating efficiency between each fraction. We thus calculated the frequency of cells that survived to form N + A clones per plating by multiplying the frequency of N + A clones by the respective plating efficiency of each fraction (Table 2C). This value (F^-:F^+:F^{2+} = 1:2.9:20) might represent a correlation between the EGFP-fluorescence intensity and the lineage potentials of the sorted cells. Taken together, these data indicate a preferential enrichment of multipotent progenitor cells in the pool of the highly E/nestin:EGFP-expressing cells.

FACS-Sorted E/nestin:$EGFP^{2+}$ Cells Self-Renew and Generate Neurons, Astrocytes, and Oligodendrocytes through Primary and Long-Term Sphere Cultures

Primary sphere formation and differentiation assay. We next examined the ability of the sorted cells to form spheres, and further tested whether the cells that showed the highest level of EGFP were multipotent and self-renewing, which are criteria for NSCs. E14 cerebral cells were fractionated by FACS, and the viable cells in each fraction were counted and plated at a density of 10^4 cells/ml. These cells were cultured for 7 days in serum-free media supplemented with 20 ng/ml each of FGF2 and EGF, then the number of neurospheres with a diameter >50 μm was counted.

We found a striking difference between the strongly E/nestin:EGFP-expressing cells and the nonfluorescent controls in the number of sphere-initiating cells harbored: Whereas 3.37% of F^{2+} cells generated spheres, only 0.42 ± 0.05% of the sorted but unfractionated controls did so (Fig. 6). Both of these proportions may have been artifactually low, because sphere formation appeared to be impeded by the relatively traumatic process of cell sorting: Sphere formation by sorted but unfractionated control cells (0.42 ± 0.05%) was only one-sixth that of unsorted controls (2.52 ± 0.31%, mean ± SD, n = 4 wells), even though the cells were viable at the time of plating. Thus, the sorting proce-

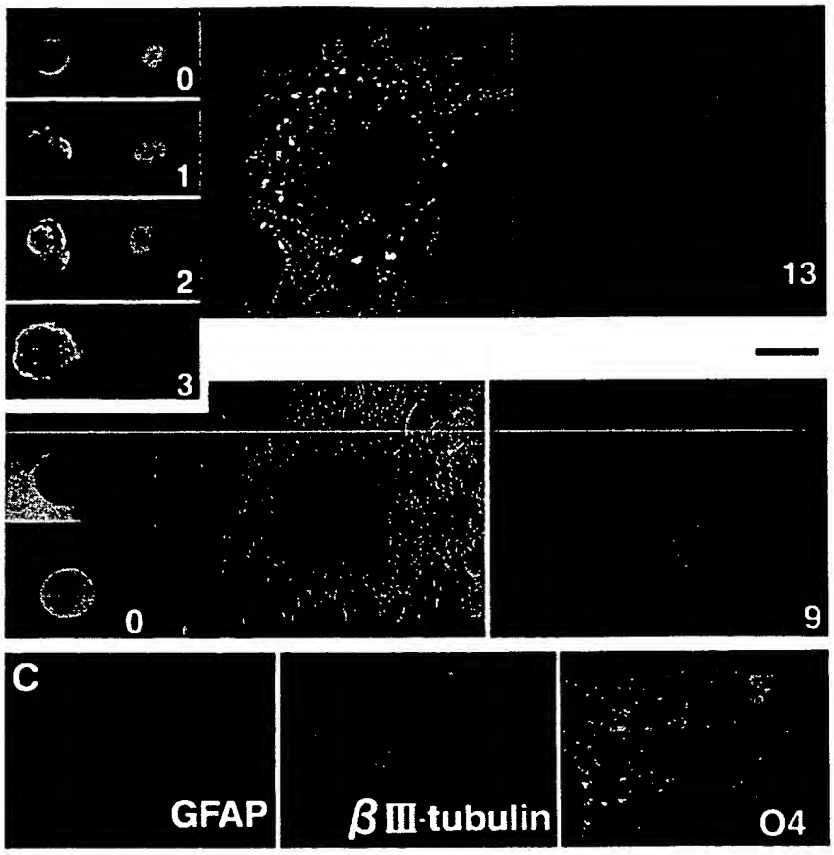

FIG. 4. Embryonic $EGFP^{2+}$ cells form neurospheres that are enriched with $EGFP^{2-}$ cells. (A) A series of time-lapse photomicrographs showing the formation of a fluorescent sphere from a single $EGFP^{2+}$ cell (the identical field is shown). The number in each pair of phase-contrast (left) and fluorescent (right) pictures indicates the day in culture. The observed field was identified using a scratch on the culture dish as a reference (not shown). (B) An EGFP fluorescent sphere, which was generated from a single $EGFP^{2+}$ cell, was transferred onto a PEI-coated dish (left), where it formed a cellular sheet by day 9 after transfer (right). Cells in the periphery of the sheet, which had spread thin, forming a monolayer, were EGFP-negative. In contrast, cells in the thicker part (the center) of the sheet still expressed EGFP. (C) In cellular sheets formed by clonally cultured spheres that were initiated by single $EGFP^{2+}$ cells, $GFAP^{-}$ astrocytes, βIII-tubulin $^{-}$ neurons, and $O4^{+}$ oligodendrocytes were generated, indicating the multipotency of the sphere-initiating $EGFP^{2+}$ cells. Bar: 25 μm in A; 210 μm in B; 20 μm in C.

dure itself may have been detrimental to cell survival and/or proliferation. Accounting for the roughly 1/6 recovery by FACS of neurosphere-competent stem cells, one might predict that the frequency of sphere-initiating cells in F^{2+} was approximately 20.0% (3.37 × 6) at E14 (Fig. 6).

We next examined the lineage potential of those sphere-initiating cells enriched in the F^{2+} fraction. To this end, we transferred spheres formed in F^{2+} cultures to PEI-coated coverslips, and cultured them as monolayers for 7–10 days. Of 27 single sphere-derived sheets, 25 (93%) developed various proportions of neurons, astrocytes, and oligodendrocytes together; 2 (7%) contained only neurons and astrocytes. Thus, cells strongly expressing E/nestin-driven EGFP (F^{2+} fraction) included sphere-initiating progenitor cells that were multipotent, which represented at least 20.0% of the cells in this fraction.

Secondary sphere culture to examine the self-renewal of the sorted cells. To examine the capacity of the sorted F^{2+} cells for self-renewal, primary spheres formed in F^{2+} culture were individually picked up and transferred to separate wells, then dissociated into single cells. These single sphere-derived cells were then cultured for 7 days and assessed for secondary sphere formation. Spheres showing EGFP fluorescence similar to the primary spheres formed in almost all cases (59/60 wells). Such passage and formation of new

TABLE 2
Characterization of Cells Fractionated by FACS Based on EGFP Fluorescence Intensity

	EGFP intensity		
	F−	F+	F++
(A) Immunocytochemistry			
%Nestin$^+$	24.9 ± 14.4	68.0 ± 15.7	98.2 ± 0.3
%βIII-tubulin$^+$	69.8 ± 12.0	37.5 ± 7.7	0.9 ± 0.3
(B) Labeling of cells with BrdU			
In vivo	3/262 (1.1%)	25/251 (10.0%)	68/251 (27.1%)
In vitro	9/289 (3.1%)	19/292 (6.5%)	58/172 (33.7%)
(C) Monolayer culture at low cell density			
Number of independent cultures	10	11	10
Plating efficiency (%)	2.8 ± 2.0	4.2 ± 3.0	11.0 ± 8.7
Frequency of clone types (%)			
N	52/71 (73.2)	77/135 (57.0)	30/221 (13.6)
A	8/71 (11.3)	18/135 (13.3)	16/221 (7.2)
N + A	11/71 (15.5)	40/135 (29.6)	175/221 (79.2)
Frequency of the cells that survived to form N + A clones (%)	0.4	1.2	8.7
Ratio	1	2.9	20

Note. FACS-sorted E14–15 cerebral cells (see Fig. 5) were subjected to immunocytochemical analysis (A), BrdU analysis (B), and low-density monolayer culture (C). (A) Cells gated into three fractions were plated onto PEI-coated coverslips, fixed immediately after they were attached, and subjected to immunocytochemical analysis. More than 300 cells were counted in each sample. (means ± SD, 3 experiments). (B) *In vivo:* E14 embryos whose mothers had been given BrdU twice (30 min apart) were removed 10 min after the second injection. Forebrain cells from the embryos were fractionated by FACS and immunostained immediately after the cells were attached to coverslips. The data presented are from one representative experiment and similar results were obtained in separate experiments with modified fractionation protocols. *In vitro:* FACS-sorted E15 forebrain cells were plated onto coverslips and cultured 12 h in DMEM/F12-based growth medium with EGF and bFGF (both 20 ng/ml) and then treated with BrdU (10 μM, 1 h) and immunostained with anti-BrdU. Data from one representative experiment are presented. Similar results were obtained in separate experiments with modified fractionation protocols. (C) E14 cerebral cells were plated onto PEI-coated plastic dishes at a low density (150 cells/0.2 cm^2 or 300 cells/0.4 cm^2) and cultured for 7 days and then fixed and stained with cell-type-specific antibodies: βIII-tubulin for neurons, and GFAP for astrocytes. The frequencies of observation of clones consisting of neurons only (N), astrocytes only (A), and neurons plus astrocytes (N + A) are presented as the percentages of total clones obtained in 10 or 11 cultures (two independent experiments). Plating efficiencies are presented as the mean ± SD from the indicated number of cultures. The frequency of cells that survived to form N + A clones per total number of cells plated was obtained by [plating efficiency] × [frequency of N + A clones].

spheres was reproduced in several independent sets of experiments, and could be repeated for more than 3 months. The capacity to give rise to neurons, astrocytes, and oligodendrocytes was also confirmed by differentiation assays using (1) F^{2+} cell-derived spheres passaged five or six times during 1–2 months (13 of 28 spheres), and (2) spheres formed by cells that were dissociated from spheres that had been passaged seven times and then—3 months after harvesting the original cells—sorted again into F^{2+} (5 of 5 spheres). These results further indicated that the original cells gated into F^{2+} could be maintained over an extended period of time, while exhibiting both multipotency and self-renewability.

E/nestin:EGFP Expression in the Adult CNS

We next examined the EGFP expression in the adult CNS of the E/nestin:EGFP-transgenic mice. By confocal microscopic observation, EGFP fluorescence was seen along the lateral ventricle (Figs. 7B–7I) and its entire caudal extension to the spinal cord (Fig. 7J). In the cerebral periventricular area, a high level of EGFP ($EGFP^{2+}$) was seen in a subset of ciliated ependymal cells, although some ependymal cells were only moderately fluorescent ($EGFP^+$) (Fig. 7B). A moderate level of EGFP was also seen in the subependyma, which was S100 negative (Fig. 7C). Such $EGFP^+$ cells in the subependyma were $Musashi1^+$ (Figs. 7D–7F). This expression pattern was apparent as early as postnatal day (P) 2 (Fig. 7A).

As adult NSCs are known to be relatively quiescent and proliferate very slowly *in vivo* (Morshead *et al.*, 1994), we also performed BrdU-labeling using two methods; one to detect cells proliferating very slowly (Doetsch *et al.*, 1999) and another to detect proliferative cells for which the cell cycle length is very short (Morshead *et al.*, 1994). By the former method (14-day label-

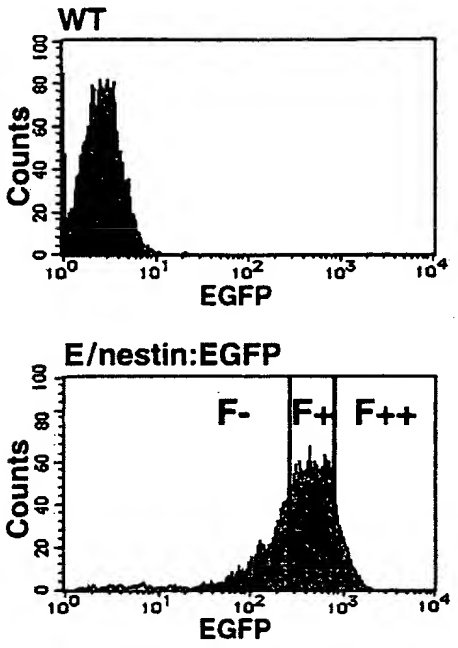

FIG. 5. Sorting of embryonic EGFP-expressing cells. FACS profiles of forebrain cells from E14 wild-type (WT) and E/nestin:EGFP mice. Anterodorsolateral cerebral fragments containing the "rostral focus" (Smart, 1983) were isolated from E14 transgenic mice. Dissociated cells were divided into F^- (30%), F^+ (60%), and F^{2+} (10%) fractions by FACS. Note that there are no breaks between the three fractions on the histogram.

ing period followed by 7-day wash out), cells in the subependyma showing a moderate level of EGFP were $BrdU^+$ (Figs. 7G and 7H). By the latter method (6-h labeling period), $BrdU^+$ cells were again detected in the subependymal zone. However, these cells, which presumably corresponded to the transiently amplifying cells (non-NSCs) (Morshead *et al.*, 1994), were negative for EGFP (Fig. 7I). No $BrdU^+$ cells were detectable in the ependyma by either labeling method.

These results strongly suggest that NSCs, or at least regions in which NSCs exist, can be identified based on EGFP expression in E/nestin:EGFP-transgenic mice.

Adult $EGFP^+$ Cells become More Fluorescent in Culture and Form "Bright" Spheres

We next examined the relationship between E/nestin:EGFP expression in adult forebrain cells and the ability of these cells to generate neurospheres in suspension culture. Cells were harvested from the forebrain periventricular area (Fig. 8A) of adult nestin-EGFP mice (three independent experiments using a total of 18 mice). EGFP-positive cells (both $EGFP^+$ and $EGFP^{2+}$) were almost always (>90%) $Musashi1^+$ and rarely $GFAP^+$ (<5%). $EGFP^{2+}$ cells were typically (>95%) $S100^+$. Some of the $S100^+$ cells (51.4 ± 10.7%, mean ± SD) and $GFAP^+$ cells (41.0 ± 7.4%) were $EGFP^+$. RC2 immunoreactivity was not detected, consistent with a previous report (Misson *et al.*, 1988) (not shown). Cells were sorted into the three (F^-, F^+, and F^{2+}) fractions by FACS (Fig. 8B), and cultured in the presence of EGF and bFGF. F^{2+} contained both $EGFP^{2+}$ and $EGFP^+$ cells. In F^{2+} cultures, brightly fluorescent spheres were formed (Figs. 8C–8E). Fluorescent spheres were also formed in cultures of F^+ cells (Figs. 8F–8H), and in such cases, the F^+ cells that were originally $EGFP^+$ became more fluorescent (usually within 24 h) and were $EGFP^{2+}$ at 4 days (Fig. 8G). The frequency of sphere formation was significantly higher in F^+ (0.31 ± 0.19%, $n = 6$ wells) than F^{2+} (0.07 ± 0.10%, $n = 8$ wells) (Mean ± SD, $P < 0.05$ by Mann–Whitney's U test). In contrast, in F^- cultures, spheres were never generated and no intensification in fluorescence was observed (not shown). The adult-derived spheres, independent of their source (F^+ or F^{2+}), generated neurons, astrocytes,

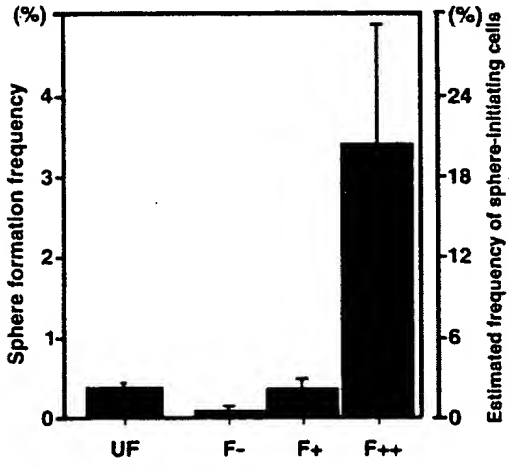

FIG. 6. Enrichment of sphere-initiating cells in the F^{2+} fraction. Fractionated E14 cerebral cells were cultured (10 cells /μl) in the presence of EGF and bFGF (both 20 ng/ml). The frequencies of sphere formation (>50 μm in diameter) per total number of cells plated were obtained at 7 days. Data represent the mean ± SD of 12 wells from two independent experiments. As controls, E14 cerebral cells that were applied to the cell sorter but not gated (unfractionated, UF) were cultured under the same conditions ($n = 4$ wells). The estimated frequency of sphere-initiating cells (right scale) was obtained with the assumption that FACS-mediated cell damage was independent of EGFP fluorescence intensity (see text).

and oligodendrocytes when transferred onto PEI-coated dishes (7/11), and generated secondary spheres when dissociated and cultured further (5/5 for primary spheres; 5/5 for secondary spheres), just as the embryonic spheres had done. More than 95% of the cells comprising these forebrain-derived spheres were Nestin$^+$, but were negative for GFAP and S100. Interestingly, of these Nestin$^+$ cells, 50–60% were strongly EGFP-positive and also RC2$^+$ (not shown). These *in vitro* cell properties were indistinguishable from those of embryonic NSCs. Thus, neurosphere propagation yielded the reemergence from adult tissue of a progenitor phenotype more typically associated with fetal brain development.

DISCUSSION

By generating E/nestin:EGFP-transgenic mice and examining the developmental properties of FACS-fractionated cells, we demonstrated a direct correlation between the level of E/nestin-driven EGFP-expression visualized in cultured cells and their ability to proliferate, self-renew, and generate both neurons and glia. Moreover, our results also suggested that different developmental properties among undifferentiated cells could be inferred in live cells based on the level of EGFP expression.

Our data also suggest that the *nestin* enhancer-driven EGFP intensity is an effective but nonexclusive marker for NSCs in the developing central nervous system. As shown in Fig. 5, histograms generated by the sorting of embryonic cerebral cells always showed a single peak with gradual tapering to both sides. This pattern suggests that the distribution of cells belonging to a given cell group may not be concentrated at a particular point (or small area) on the x-axis (EGFP intensity) of the FACS histogram. Other cell groups may be somewhat broadly distributed in a similar way. Thus, even within a cell population that had been categorized in our judgement as EGFP^{2+}, the level of EGFP expression may not be uniform. This may also be the case for F$^-$, F$^+$, and F^{2+}. This might be explained in part by the accumulation and stability of the EGFP, and by possible stochastic fluctuation of the transcriptional activation of the *nestin* second intronic enhancer. Although EGFP is advantageous for visualizing the properties of live cells, for the further purification of NSCs or lineage-restricted progenitor cells, it might be useful to sort cells by FACS using EGFP in combination with other cell surface markers.

We also cannot exclude the possibility that EGFP-

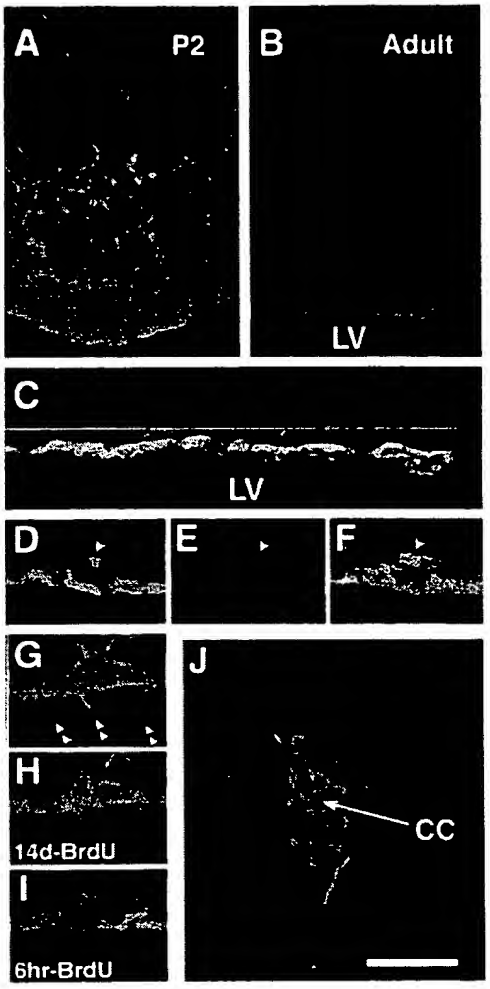

FIG. 7. EGFP expression in the postnatal and adult CNS. (A) Striatum at P2. EGFP expression was seen in the periventricular area, which contained EGFP^{2+} ependymal or process-bearing, tanycyte-like cells and EGFP$^+$ subependymal cells. (B) Adult striatum. EGFP fluorescence was seen in a segmental-like pattern, with EGFP^{2+} cells in the ependyma and EGFP$^+$ cells in both the ependyma and the subependyma. LV, lateral ventricle. (C) Merged image of anti-S100 immunostaining (red) and EGFP fluorescence (green) in the adult periventricle. S100-negative EGFP$^-$ cells were seen in the subependyma. (D–F) A magnified view, showing subependymal EGFP$^+$ cells (D) (arrowhead) were Musashi1$^+$ (red, E). F, a merged view of the data in D and E. (G) A cell in the adjacent subependymal zone (arrow) also expressed EGFP, but at a lower level (EGFP$^-$). Double-arrowheads indicate cilia. (H) An EGFP$^+$ subependymal cell (arrow) that had incorporated BrdU that was administered for 14 days, followed by a 7-day washout period, to label slowly proliferating cells (Doetsch *et al.*, 1999). (I) Short BrdU administration (6 h, 4 injections with 2-h intervals) did not label cells showing detectable levels of EGFP. Red, BrdU$^+$ nuclei. (J) In the spinal cord, EGFP fluorescence was confined to the ependyma encircling the central canal (CC). Among ependymal cells, however, the level of EGFP expression was not homogeneous, similar to the case in the forebrain. Bar: 40 μm in A and B; 35 μm in C and J; 30 μm in D–I.

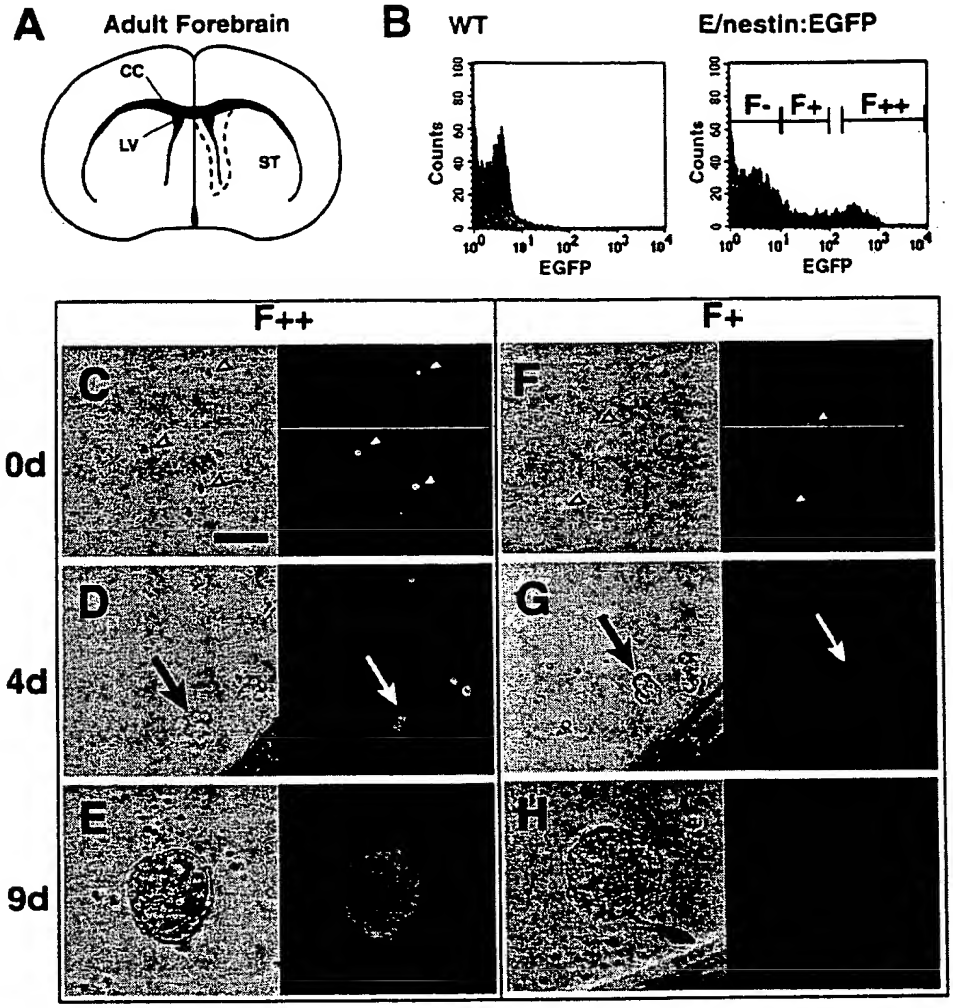

FIG. 8. Sorting of adult EGFP-expressing cells and sphere formation assay. (A) Schematic diagram of a frontal section of adult forebrain showing the dissected periventricular area (red broken lines). CC, corpus callosum. ST, striatum. LV, lateral ventricle. (B) FACS profiles of cells harvested from the forebrain periventricular area of wild-type (left) and E/nestin:EGFP (right) adult mice. Transgenic cells were sorted into three fractions (F^-, F^+, and F^{2+}). Note the gap between F^+ and F^{2+}. (C–E) Sphere formation by sorted F^{2+} cells. The indicated cells (arrowheads) at 0 day (C) showed an extremely high level of EGFP ($EGFP^{2+}$), whereas the remaining cells were moderately fluorescent ($EGFP^+$). By 4 days (D), small fluorescent cell aggregates (arrow) had formed. By 9 days (E), fluorescent spheres had formed. These spheres generated neurons, astrocytes, and oligodendrocytes on PEI-coated coverslips (see text). (F–H) Intensified fluorescence and sphere formation in F^+ cultures. Initially, the level of EGFP expression in F^- cells was "$EGFP^+$" (arrowheads in F) or lower. However, cells with more intense fluorescence appeared during culture (usually within 24 h) and their level of EGFP expression was almost equivalent to that of the $EGFP^{2+}$ cells in the F^{2+} culture at 4 days (G). Cell divisions had occurred by this point, and small aggregates were found (arrow). By 9 days (H), spheres as brightly fluorescent as those in the F^{2+} culture had formed. These spheres exhibited a similar differentiation potential as spheres formed in the F^{2+} culture. In the F^- culture, spheres were never generated and no intensification of fluorescence was observed (not shown). Bar: 50 μm in C–H.

negative multipotent progenitor cells exist in the embryonic brain, because spheres also formed in cultures of F^- cells (0.08 ± 0.08%) (Fig. 6). However, compared with the frequency of sphere-initiating cells in F^{2+} cells (F^-:F^{2+} = 1:42.1), such EGFP-negative stem cells are rare, even if they do exist.

In the adult E/nestin:EGFP-transgenic mice, it appears that the population of E/nestin:EGFP-defined cells include slowly dividing multipotential progenitor cells, which may be viewed as putative adult stem cells. This conclusion is based on the following results: (1) EGFP expression was confined to the periventricular

region, the likely reservoir of adult neural stem and progenitor cells (Luskin, 1988; Lois and Alvarez-Buylla, 1993; Morshead *et al.*, 1994; Pincus *et al.*, 1998), (2) EGFP-expressing cells harvested from the periventricular regions of the E/nestin:EGFP-transgenic mice displayed neurosphere-forming activity (Reynolds and Weiss, 1992, 1996), while EGFP-negative cells did not, and (3) slowly dividing cells, which may be candidates for NSCs, showed a moderate level of EGFP expression, while rapidly dividing cells in the subependyma (non-NSCs) (Morshead *et al.*, 1994) were negative for EGFP expression. As regards the origin of NSCs in the adult forebrain, Johansson *et al.* (1999) found that ependymal cells exhibit the cellular properties of stem cells. Doetsch (1999) and Chiasson *et al.* (1999) then used different methodologies to conclude that at least some subependymal cells may be adult NSCs. Here we showed that a subset of both ependymal and subependymal cells expressed EGFP fluorescence at heterogeneous intensities in E/nestin:EGFP-transgenic mice. Although our *in vitro* examinations of adult forebrain periventricular cells showed that sphere formation occurred more frequently in F^+ than in F^{2+}, the heterogeneous EGFP expression pattern in the ependyma and subependyma prohibited us from determining which cell types in this area (ependymal cells or subependymal cells) predominated in the formation of the bright spheres. Further studies addressing the potential heterogeneity of E/nestin:EGFP-positive cells in the adult CNS using different approaches (e.g., Chiasson *et al.*, 1999; Doetsch *et al.*, 1999) are needed.

A particularly interesting finding was that moderately EGFP-expressing cells that were sorted into the F^+ fraction gained EGFP intensity during culture (within 24 h) (Fig. 8G). $EGFP^{2+}$ cells were highly enriched in the spheres derived from the adult CNS. These *in vitro* data are consistent with the previous *in vivo* findings that EGF infusion into the adult mouse forebrain increases the total amount and spatial distribution of Nestin staining (Craig *et al.*, 1996). Thus, *nestin* enhancer in the adult NSCs is likely to be activated in the presence of EGF and bFGF *in vivo* and *in vitro*. Taken together with the developmental changes in the correlation between EGFP intensity and the frequency of sphere formation, these results suggest that adult CNS stem cells exist in a different microenvironment *in vivo* from that of the embryonic CNS stem cells, with respect to the presence of growth factors. Such environmental differences might be one reason for the different proliferative kinetics of the adult and embryonic CNS stem cells *in vivo* (Morshead *et al.*, 1994; Martens *et al.*, 2000).

In summary, by combining FACS and functional analyses, we have characterized the EGFP-expressing cells of transgenic mice expressing EGFP under the regulatory control of the *nestin* enhancer. We have also obtained enriched populations of cells that are multipotent and self-renewing, definitive criteria for NSCs, from the developing murine CNS. Thus, this mouse line provides a useful tool for future efforts to dissect the molecular mechanisms regulating both the self-maintenance and phenotypic diversification of these cells *in vitro* and to analyze the biology of NSCs in their *in vivo* environment.

EXPERIMENTAL METHODS

Transgenic Mice

An evolutionarily conserved 637-bp (1162–1798) second intronic region of the rat *nestin* gene (Lothian and Lendahl, 1997) (from the Xh5 plasmid) was subcloned into the *Sma*I site of the hsp68-EGFP plasmid containing a 1.0-kb *Nru*I–*Bam*HI fragment of the minimum promoter of heat shock protein 68 (hsp68) (from the Ass-hsp68-*lacZ*-pA vector) and a 1.0-kb *Nru*I–*Bam*HI fragment containing the EGFP coding region and a polyadenylation signal from the pEGFP-N3 (Clontech Laboratories). The 2.7-kb nestin-hsp-EGFP fragment (*Sal*I) was purified, then injected into the pronucleus of fertilized mouse eggs. The minimum promoter of heat shock protein 68 (hsp68) exhibits no basal activity unless an enhancer is placed in its vicinity (Rossant *et al.*, 1991). The base numbering of the rat *nestin* gene coincides exactly with the GeneBank submission, Accession No. AF004334. Six independent lines showing a similar expression pattern were established in a C57BL/6 background. The data presented were obtained from experiments using heterozygous mice from a single strain (#25). All procedures were performed in accordance with institutional guidelines. For timed pregnant mice, the date the vaginal plug was observed was defined as day 0.

Cell Preparation and Sorting

Brain tissues were dissected from transgenic or wild-type mouse embryos in DMEM/F12 (GIBCO), then digested in 0.25% trypsin (GIBCO) at 37°C for 5 min. After quenching the digestion with medium containing ovomucoid inhibitor (0.7 mg/ml, Sigma), the material was triturated with a fire-polished Pasteur pipette. Fragments of the adult forebrain periventricular area were enzymatically digested as described previously

(Reynolds and Weiss, 1992). Dissociated cells were suspended in PBS containing 10 μg/ml propidium iodide (PI), and filtered (30 μm). Cell sorting and analyses were performed using a FACS Vantage flow cytometer/cell sorter equipped with CELLQuest software (Becton-Dickinson). Cells ($1-2 \times 10^6$/ml) were analyzed for forward scatter, side scatter, PI fluorescence, and EGFP fluorescence with an argon laser (488 nm, 100 mW) (Wang *et al.*, 1998). Dead cells were excluded by gating on forward and side scatter, and by eliminating propidium iodide-positive events. Viable cells from the transgenic mice were sorted into DMEM/F-12 medium at a speed of 3000 events/s. Sorted cells were kept on ice until used for further experiments.

Cell Culture

For sphere cultures, sorted or unsorted cells were washed twice with DMEM/F12 and resuspended in DMEM/F-12-based serum-free growth medium containing insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), sodium selenate (30 nM), putrescine (60 μM), EGF (20 ng/ml), and bFGF (20 ng/ml) (all from Sigma) (Reynolds and Weiss, 1996). A 1:1 cocktail of this cell suspension and neurosphere conditioned medium was plated into each well at 10 cells/μl, lower than the cell density at which virtually all spheres were clonal (Hulpas *et al.*, 1997). Spheres that formed by 7–10 days *in vitro* were plated onto polyethylenimine (PEI)-coated coverslips and cultured further for 7–10 days in DMEM/F12 containing 1% FBS and 10^{-6} M 13-cis-retinoic acid (Nakarai, Japan). Seven-day-old spheres were mechanically dissociated into single cells, and the cells were cultured further for sphere formation.

For monolayer cultures, the culture surface of PEI-coated plastic dishes was divided into separate zones by means of three silicone rubber rings (5–7 mm inner diameter, 1 mm thick), outside of which astrocytes from perinatal wild-type mice were seeded as a feeder cell layer (Miyata and Ogawa, 1994). When astrocytes had formed a confluent monolayer, sorted cells (F^-, F^+, and F^{2+}) or unsorted cells suspended in the DMEM/F12-based growth medium supplemented with 2% FBS were individually plated onto the three circular areas (coated with 10 μg/ml fibronectin, Sigma) at a density of 750 cells/cm^2. Just before plating, single cell dissociation (>98%) was confirmed under an inverted microscope (20×). After cells attached to the dishes (within 1 h), the silicon rings were removed, and fields containing clusters of >1 cell were excluded from analysis. Sorted cells were damaged more often than unsorted cells even though all cells had been viable at the time they were plated. Since most cell death occurred within 24 h in the low-density cultures of the sorted cells, the actual cell density on the next day was estimated to be <100 cells/cm^2 for the sorted cells. The possibility of feeder cells contaminating the fields of interest was excluded by daily observation. Half the medium was renewed every 2–3 days, and cells were fixed on day 7 or in some cases on days 1 and 3 (unsorted cells). Cells were observed under an inverted fluorescent microscope (IX70, Olympus, Japan) equipped with a cooled CCD digital camera (SPOT2, Seki Technotron, Japan), using an FITC filter set. Despite the reported stability of EGFP in mammalian cells, we observed a rapid disappearance of E/nestin:EGFP when cells were allowed to differentiate (within 12–24 h) (Figs. 3A, 3B, and 4B).

Immunochemistry

Frozen sections (12 μm) of brains fixed with 4% paraformaldehyde were prepared as described previously (Sakakibara *et al.*, 1995). Unsorted or sorted cells were attached to PEI-coated coverslips, then fixed in 4% paraformaldehyde. Cellular sheets formed by spheres were fixed similarly. Cells were then stained with mouse anti-Nestin (Rat401, Hybridoma Bank), anti-βIII-tubulin (Sigma), anti-MAP2 (Sigma), anti-GFAP (rabbit, DAKO or mouse IgG, Sigma), O4 (mouse IgM, Boehringer-Mannheim), anti-Mushashi1 (rat IgG), RC2 (mouse IgM, from Dr.Yamamoto, Tsukuba Univ., Japan), anti-S100 (rabbit, MEDAC), anti-PSA-NCAM (mouse IgM, from Dr. Seki, University of Juntendo, Japan), and anti-GFP (rabbit, Clontech), as described (Kaneko *et al.*, 2000). EGFP was readily detected in fixed specimens but was more bleached than in live cells. Anti-GFP did not show nonspecific staining, and was used for double or triple staining. Live cells that were judged as $EGFP^{2+}$ were almost always (98–100%) immunochemically $EGFP^{2+}$. Specimens were examined using a confocal microscope (LSM510, Carl Zeiss) or a universal fluorescent microscope (Axiophoto 2, Carl Zeiss). Digital images were captured with a CCD camera (FUJIFILM HC-200 equipped with Photograb II software).

BrdU Labeling

FACS-sorted cells were suspended in medium containing EGF and bFGF, plated onto PEI-coated coverslips (1×10^5 cells/cm^2), and incubated overnight. They were then treated with BrdU (10 μM) for 1 h, fixed in 4% paraformaldehyde, and immunostained with anti-

GFP first, followed by FITC-conjugated secondary antibody. Cells were then refixed with the same fixative, pretreated with 2 N HCl containing 0.01% Triton X-100, and incubated with anti-BrdU (mouse IgG, 1:200; Sigma), followed by rhodamine-conjugated secondary antibody.

For labeling *in vivo*, timed pregnant female mice bearing nestin-EGFP transgenic embryos were injected twice with BrdU (0.1 mg/g body weight of the pregnant mouse for each injection, 30 min apart) and the embryos were isolated 10 min after the second injection. Forebrain cells were harvested from the BrdU-treated embryos, sorted by FACS, then plated onto coverslips. One hour later, the cells were fixed and immunostained with anti-BrdU. To label slowly dividing cells in the adult brain, BrdU (1 mg/ml) was given to mice in their drinking water for 2 weeks, and the cells were harvested 1 week later. To label rapidly dividing cells in the adult brain, BrdU was administered four times by intraperitoneal injection (0.1 mg/g body weight, 2 h apart, 6 h total labeling time).

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